# Plant defence responses in cassava (Manihot esculenta Crantz) suspension cells challenged with elicitors

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# PLANT DEFENCE RESPONSES IN CASSAVA (*Manihot esculenta* **Crantz**) SUSPENSION CELLS CHALLENGED WITH ELICITORS

Submitted by
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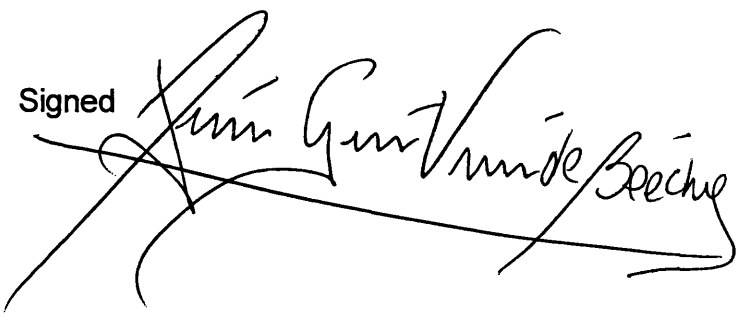
Signed 





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**I dedicate this thesis to the memory of my Mother**

**Esther Vásquez Restrepo de Gómez**

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# ABSTRACT

Cassava (*Manihot esculenta* Crantz) is a key crop for the poorest countries in the World. Despite its importance as staple food, research on this plant has been neglected for many years. Several pathogens can affect cassava, but principally, yield production is severely affected by the two diseases cassava bacterial blight (CBB) and African cassava mosaic virus (ACMV). However, the mechanisms of resistance in this plant to any pathogen are not well understood. Plants defend themselves from attempted infection and other environmental stresses by the orchestrated release, activation and synthesis of a range of enzymes, low molecular weight compounds and reactive oxygen species. In order to study components of these defence responses in cassava, a cell-suspension-culture / elicitor system was established using yeast cell wall glucan which was chosen from several as an effective elicitor.

The earliest detected defence responses in plants are changes in medium pH and activation of an oxidative burst resulting in production of reactive oxygen species (ROS). A rapid, transient oxidative burst was detected as $H_2O_2$ that peaked (10.5 μM) within 5-10 minutes after elicitation. A possible source of this oxidative burst in plants is xanthine oxidoreductase (XO). To date no XO has been

cloned in plants. Therefore attempts were made to clone XO from cassava using a degenerate primer PCR approach, however these were not successful. XO activity was constitutive and did not increase significantly following elicitation.

Another possible source of ROS is peroxidase. In elicitor-challenged cassava cells peroxidase (POD) activity increased 4-fold 48 h following elicitation. By contrast in the spent medium of cells, peroxidase decreased after 12 h following elicitation whereas in non-elicited cells extracellular peroxidase had increased 4-fold by 48 h. Gene expression of a cassava peroxidase (MecPOD1) was examined by northern blots and showed an increase in transcripts from 12 to 48 h following elicitation. POD may also play a key role in defence *via* cross-linking of the cell wall protein extensin (HRGP). Northern analysis showed a similar pattern of expression for both POD and HRGP with a peak at 24 h after elicitation in both cells and leaves. In contrast, northern blots using a cassava catalase probe (MecCAT1) in elicitor-treated cells showed down-regulation from 3 h after elicitation followed by a recovery of transcription at 24 h. Similarly in elicitor-treated leaves catalase transcript accumulation was down-regulated from 3 h following elicitation with recovery at 24 to 48 h.

POD isoforms (2-7 isoforms, pI 3.1-8.8) in elicited and unelicited cells, extracellular medium and leaves were detected on IEF gels. At least 1 isoform (pI 3.6) was common to all samples. A single isoform (pI 3.4) was markedly enhanced following elicitation in the extracellular medium at 48 h.

Scopoletin is a common hydroxyfuranocoumarin that has been described both as a phytoalexin and a phytoanticipin. The previous described POD isoform

(pI 3.6) exhibited high activity towards scopoletin in IEF gels. Non-enzymic antioxidants in both cassava cells and leaves (elicited and non-elicited) were detected on HPTLC plates with the free radical DPPH.

Phenylalanine ammonia-lyase (PAL) catalyses the first committed step of general phenylpropanoid metabolism, end products from which can play key roles in defensive responses in plants, such as cell wall strengthening, anti-microbial production and signalling. PAL increased in activity during elicitation and was maximal at 15 hours; a peak of the corresponding mRNA preceded this at 9 hours. Northern blot analysis showed that elicitor-induced transcription was dose dependent and started as early as 30 min following elicitation and could be markedly potentiated by 24 h salicylic acid pre-treatment. Phenolic products of phenylpropanoid metabolism, including scopoletin, scopolin, rutin, and kaempferol-3-*O*-rutinoside, were detected by HPLC in both cassava cells and leaves (elicited and non-elicited). Detection and quantification of these phenolic compounds was made by HPLC and UV comparison with commercial standards. The phenolic compounds were tested *in vitro* for fungitoxicity against different cassava phytopathogenic fungi such as *Fusarium solani*, *Fusarium oxysporum* and the saprotroph *Trichoderma harzianum*. Germ tube elongation was affected more markedly than germination in these fungi by esculetin, ferulic acid, quercetin and scopoletin when $EC_{50} \geq 50$ μg/ml. Ferulic acid inhibited germination and germ tube elongation in all three fungi ($EC_{50} \geq 50$ and 200 μg/ml, respectively). However, there was no inhibition by any of the phenolics of mycelial development of the fungi tested even at the highest concentration tested (1000 μg/ml). Bioassays were

performed with cassava cell extracts separated in HPTLC plates and tested against *T. harzianum* and *F. avenae* and different inhibition zones were detected. The oxidation of esculetin by peroxidase and tyrosinase increased toxicity to *T. harzianum* and oxidation by peroxidase enhanced the fungitoxicity of scopoletin towards *F. solani*. The levels of phenolics detected were, in theory, too low in cells to contribute to inhibition of pathogens. However, in combination and especially in their oxidised states they may contribute to defence.

The potential contributions to defence of the multi-layered, antimicrobial components described above are considered

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# CHAPTER 5 PHENYLPROPANOID METABOLISM IN ELICITOR-CHALLENGED CASSAVA

# ABBREVIATIONS

| | |
|---|---|
| **2,4-D** | 2,4-dichlorophenoxyacetic acid |
| ***AA*** | Arachidonic acid |
| **AA** | 4-aminoantipyrine |
| **AC** | Adenylate cyclase |
| **ACC** | 1-aminocyclopropane-1-carboxylate oxidase |
| **ACMV** | African cassava mosaic virus |
| **AFLP** | Amplified fragment length polymorphism |
| **AGPs** | Arabinogalactan proteins |
| **AOPP** | α-aminooxi-β-phenylpropionic acid |
| ***avr*** | Avirulence genes |
| **BA** | Benzoic acid |
| **BAC** | Bacterial artificial chromosome |
| **BAG** | Benzoic acid glucoside |
| **BA2H** | Benzoic acid-2 hydroxylase |
| **BSA** | Bovine serum albumin |
| **BTH** | Benzo [1,2,3]-thiadiazole-7-carbothioic acid S-methyl ester |
| **CA** | Cinnamic acid |
| **CAD** | Cinnamyl alcohol dehydrogenase |
| **CBB** | Cassava bacterial blight |
| **cfu** | Colony forming units |
| **CHES** | (2-[N-Cyclohexylamino]ethanesulfonic acid) buffer |
| **CHS** | Chalcone synthase |
| **CIAT** | Centro Internacional de Agricultura Tropical |
| **CWDE** | Cell wall degradative enzymes |
| **CWP** | Cell-wall-bound peroxidase |
| **DDPH** | 1,1-diphenyl-2-picryl-hydrazyl |
| **DHBS** | (3,5-dichloro-2-hydroxybenzensulfonic acid) |
| **Dhp** | Prolyl-hydroxylase, 3,4-dehydro-L-proline |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **DTT** | Dithiothreitol 99% |
| **E** | Elicitor |
| **ECM** | Exocellular matrix |
| **EDTA** | Ethylene diamine tetra-acetic acid |
| **EFE** | Ethylene-forming enzyme |
| **EPA** | Eicosapentaenoic acid |
| **EPS** | Extracellularpolysacharide |
| **$E_r$** | Receptor |

| | |
|---|---|
| **FDA** | Fluorescein diacetate |
| **FITC** | Fluorescein isothiocyanate |
| **FPLC** | Fast performance liquid chromatography |
| **FW** | Fresh weight |
| **G** | GTP-binding protein(s) |
| ***Gga*** | *Gaeumannomyces graminis* var. *avenae* |
| **GP** | Gluthatione peroxidase |
| **GRP** | Glycine-rich protein |
| **GST** | Gluthatione S-transferase |
| **HMK** | (+)-6a-hydroxymaackiain |
| **HPDase** | Hydroxyperoxide dehydrase |
| **HPLC** | High performance liquid chromatography |
| **HP-TLC** | High performance thin layer chromatography |
| **HR** | Hypersensitive response |
| **HRGP** | Hydroxyproline-rich glycoprotein |
| **HRP** | Horseradish peroxidase |
| **$HO_2^-$** | Hydroperoxyl radical |
| ***hrp*** | Hypersensitive response and pathogenicity genes |
| **IAA** | Indoleacetic acid |
| **INA** | 2,6-dichloroisonicotinic acid |
| **LB** | Luria broth medium |
| **LPS** | Lipopolysaccharide |
| **LRR's** | Leucine rich repeats |
| **Lys** | Lysine amino acid |
| **MES** | (2-[N-morpholino] ethanesulfonic acid) |
| **MS** | Murashige and Skoog medium |
| **MTT** | 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide |
| **MJ** | Methyl jasmonate |
| **MW** | Molecular weight |
| **NAA** | Naphthaleneacetic acid |
| **NA** | Nutrient agar |
| **NYGB** | Nutrient yeast glycerol broth medium |
| **OD** | Optical density |
| **OGA** | Oligalacturonic acid |
| **OGA-R** | Oligalacturonide fragments and receptor |
| **$^{\cdot}OH$** | Hydroxyl radical |
| **$^{\cdot}O_2^-$** | Superoxide anion |
| **p** | Phosphatase |
| **PAL** | Phenylalanine ammonia-lyase |
| **PCR** | Polymerase chain reaction |
| **PCV** | Packed cell volume |
| **PDA** | Potato dextrose agar |
| **Pgase** | Polygalacturonase |
| **PGIP** | Polygalacturonase-inhibiting protein |
| **Phe** | Phenylalanine |

| | |
|---|---|
| **PLase A** | Phospholipase A |
| **PLase C** | Phospholipase C |
| **PMSF** | Alpha-toluenesulfonyl fluoride 99% |
| **POD** | Peroxidase |
| **PPD** | Postharvest physiological deterioration |
| **PR** | Pathogenesis-related gene |
| **PrP or P/HRGP** | Proline-rich protein |
| **QTL** | Quantitative trait locus |
| ***R*** | Resistance gene |
| **RFLP** | Restriction length polymorphism |
| **Rp** | Plant receptor protein |
| **RP-HPLC** | Reverse phase high performance chromatography |
| **RNA** | Ribonucleic acid |
| **ROS** | Reactive oxygen species |
| **SA** | Salicylic acid |
| **SAG** | Salicylic acid glucoside |
| **SAR** | Systemic acquired resistance |
| **SDS** | Sodium dodecyl sulphate |
| **SDW** | Sterile distilled water |
| **Ser** | Serine amino acid |
| **SOD** | Superoxide dismutase |
| **TCA** | Trichloroacetic acid |
| **TFA** | Trifluroracetic acid |
| **TLC** | Thin layer chromatography |
| **TMV** | Tobacco mosaic virus |
| **TTC** | 2,3,5-triphenyl tetrazolium chloride |
| **Tyr** | Tyrosine amino acid |
| **Val** | Valine amino acid |
| ***Xac*** | *Xanthomonas cassavae* pathovar *cassavae* |
| ***Xam*** | *Xanthomonas axonopodis* pathovar *manihotis* |
| **XOMT** | S-adenosyl-L-methionine:xanthotoxol O-methyltransferase |
| **XO** | Xanthine oxidase |
| ***Xoo*** | *Xanthomonas oryzae* pathovar *oryzae* |

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# CHAPTER 1

# GENERAL INTRODUCTION

## 1. CASSAVA

### 1.1 BOTANICAL DESCRIPTION

Cassava (*Manihot esculenta* Crantz) is a dicotyledonous perennial plant, which belongs to the *Euphorbiaceae* family. Other members of this family include important crop plants such as rubber tree (*Hevea brasiliensis*), and castor bean (*Ricinus communis*) (Cock 1985). Like many members of the family, cassava contains laticifers, which produce latex. It is a cyanogenic plant, and along with other members of the genus *Manihot*, has a chromosome number 2n=36 and polyploids are not common (Onwueme 1978).

The cassava plant grows as a woody shrub **(Figure 1.1)**. Depending on the cultivar, cassava can grow to a height of 1 to 4 metres. Stems ramify at different heights with three secondary ramifications (Montaldo 1972). The colour of the mature stem varies from green to dark-brown and the diameter varies from 2 to 6 cm according to the variety.

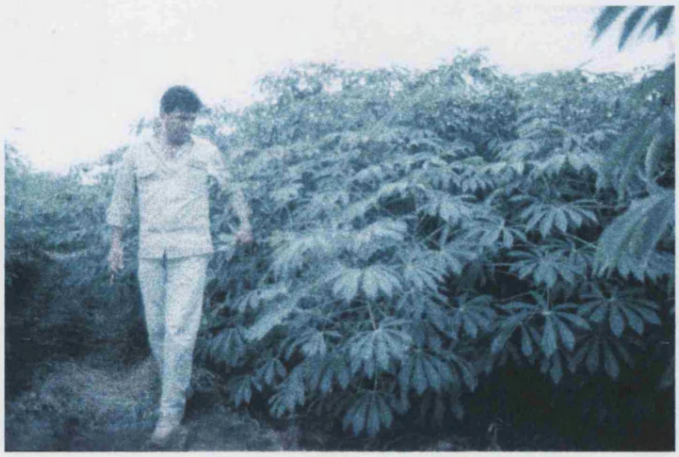

**Figure 1.1 Field grown cassava plants in Cali, Colombia.**

Leaves have a palmate leaf blade shape and are arranged in alternate spirals attached to elongated petioles **(Figure 1.2)**. The phyllotaxis is 2/5 and each leaf is subtended by 3-5 stipules. The number of lamina lobes is usually 5-7, although this may vary even between different leaves on the same plant. Venation in each lobe is reticulate. Differences in the colour of the leaves and the petioles are determined by the variety, varying from green to reddish-brown.

Flowering presents enormous differences in time and quantity of the flowers produced between varieties; some varieties do not flower at all. Flowers are borne on terminal panicles, with the axis of the branch continuous with that of the panicle inflorescence. The flowers are unisexual and both male and female flowers occur on the same plant; cross-pollination is the rule **(Figure 1.3)** (Onwueme 1978).

**Figure 1.2 Cassava leaves in University of Bath glasshouse, U.K.**

**Figure 1.3 Cassava flowers in Cali, Colombia.**

Ovoid ellipsoidal seeds 10 mm long, 5 mm wide and 4 mm deep are produced **(Figure 1.4)**. The seed has a brittle testa, which is mottled grey with black dots. Although the plant can be grown from seed, as a crop it is usually reproduced vegetatively from stem cuttings (Onwueme 1978).


**Figure 1.4 Cassava seeds in Cali, Colombia.**

Cassava roots accumulate carbohydrates in the parenchyma to form swollen storage organs **(Figure 1.5)** (Domínguez et al. 1985). A mature cassava root may range in length up to 100 cm and in weight from 0.5-2.0 kg, depending on variety and growing conditions. The root surface varies according to the cultivar, but is predominantly brown. The internal structure can be classified into three regions **(Figure 1.6)**. The outermost layer is the periderm, composed mostly of dead cork cells, which along with the cortex constitutes the peel. The central portion of the storage root, is the flesh, which consists mostly of parenchymatous cells containing large amounts of stored starch. Latex tubes (laticifers) occur in the flesh of the storage root, and also in

the cortex and in other parts of the plant. Finally, there is the central vascular strand (Onwueme 1978).

**Figure 1.5 Cassava roots in Bogor, Indonesia.**

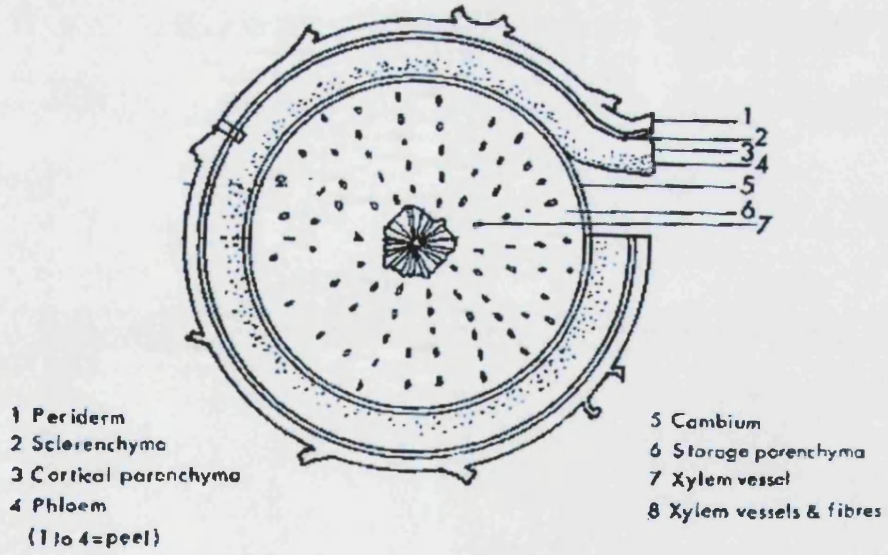


**Figure 1.6 Cassava root structures (from Hunt et al. (1977)).**

## 1.2 ORIGIN, DISTRIBUTION AND PRODUCTION

All species of the genus *Manihot* (around 98 different species) are from the American continent where there are two main areas of species diversity: one in Mexico and Northern Central America, and the other from Paraguay to Northern Brazil (León 1968; Rogers & Appan 1973). There are also two centres of diversity of cassava in America, in the Central America-Mexico region and another in Brazil, suggesting a possible dual origin for the domesticated crop (Allem 1987; Rogers & Appan 1973). Based on phylogenetic analysis, using microsatellite-primed polymerase chain reactions (PCR) markers, it was suggested that the South American cassava subspecies *M. flabellifolia* and *M. peruviana* are the closest wild relatives of cassava (Allem 1987; Roa et al. 1997). A recent phylogenetic study, based on the single-copy nuclear gene, glyceraldehyde 3-phosphate dehydrogenase, concludes that cassava was probably domesticated from wild *M. esculenta* populations of subspecies *flabellifolia* along the southern border of the Amazon basin (Olsen & Schaal 1999).

Since prehistoric times, cassava has been an important crop in the tropics. Piperno & Holst (1998) reported the presence of starch grains, similar in form to those from contemporary cassava roots, on prehistoric stone tools in Panama (Central America) dated as 8,000 years old. Furthermore, in Mexico, cassava starch has been identified in human coprolites that are 2,100 to 2,800 years old (Cock 1985).

Spanish conquistadors found cassava as a dietary staple food on the islands of the Caribbean as well as on the continent. It was one of the few indigenous crops readily adopted by the Spaniards; Cortéz took large quantities

of cassava flour with him from Cuba for his conquest of Mexico (Thomas 1992). Portuguese sailors introduced the plant to Africa in the late sixteenth century from Brazil (Cock 1985). It is probable that multiple introduction of cassava occurred *via* several ports on the West Coast of central Africa. By the middle of the eighteenth century cassava was introduced into East Africa and Madagascar. At the beginning of the eighteenth century, cassava was introduced to Goa on the Indian subcontinent. Probably at the same time, it was transported to Indonesia and the Philippines from Mexico, and by the nineteenth century, cassava was widely grown in South and Southeast Asia. The Chinese in 1906 developed a trade in "tapioca" to Europe.

Cassava is a staple root crop for over 500 million people living throughout the tropics. For example, in sub-Saharan Africa, jungle regions of Bolivia, Peru, Ecuador, on the north coast and in the Santander Department of Colombia, and in the rural areas of many Caribbean islands, cassava is the primary source of carbohydrates. It is important in the diet of most areas of the lowland tropics of Africa and is a staple food in Democratic Republic of Congo, Tanzania, Mozambique, and Madagascar (Cock 1985).

Today, cassava is cultivated in the tropical and subtropical areas of the world. Cassava is grown in approximately 90 different countries at altitudes between sea level and 2,000 metres, between 30°N and 30°S, and under diverse edaphological and ecological conditions. Generally, this crop is still cultivated by small-scale farms using low-level technology. For some countries, especially in West Africa, cassava is the most economical, lowest risk, subsistence crop for the small-scale farmer due to its capacity to adapt to poor soils and dry conditions (Bellotti & van Schoonhoven 1978). Nevertheless, the

best conditions in which to grow cassava is in light sandy loam soil of medium fertility with good drainage (Onwueme 1978).

Economically, the most important part of the plant is the roots. Many people consume as much as one kilogram of cassava root products daily. Leaves are also used as food; these are much richer in protein and vitamins than the roots **(Table 1.1)** (Onwueme 1978).

| Content in 100 g fresh weight | | |
|---|---|---|
| | **Roots** | **Leaves** |
| Water | 62 g | 80 g |
| Carbohydrate | 35 g | 7 g |
| Protein | 1-2 g | 6 g |
| Fat | 0.3 g | 1 g |
| Calcium | - | 0.2 g |
| Iron | - | 0.3 g |
| Vitamin $B_1$ (thiamine) | Trace | 0.2 mg |
| Vitamin $B_2$ (riboflavin) | Trace | 0.3 mg |
| Vitamin C | 35 mg | 200 mg |
| Vitamin A | Trace | 10,000 IU |
| Niacin | Trace | 1.5 mg |

**Table 1.1 Important components in cassava roots and leaves (after Onwueme 1978).**

Cassava storage roots are also used to produce concentrate for animals, for the production of starch, and paper and for some industrial processes such as alcohol fermentation, and acetone production (CIAT 1992; Cock 1985;

Cooke & Cock 1989). For instance, Thailand and Indonesia export cassava to Europe as concentrate food for animals and for starch extraction (Henry 1992).

Depending on the variety and ecological conditions, yield production can be as much as 23 tons per hectare. The leading producers in the world are Brazil, Nigeria, Democratic Republic of Congo, and some Asian countries **(Table 1.2)**. World annual production of cassava was about 165 millions tons per annum between 1995 and 1997. Between 1993 and 1995 the world usage was 59% as food, 24% as animals feed and 17% for industrial purposes (CIAT 1992; FAO 2000).

| | **1995** | **1996** | **1997** |
|---|---|---|---|
| | **(Million tonnes)** | | |
| **World** | 165.2 | 164.8 | 166.4 |
| **Africa** | 84.9 | 84.2 | 85.2 |
| **Asia** | 48.2 | 48.8 | 48.6 |
| **LA & C*** | 31.9 | 31.6 | 32.4 |

**Table 1.2 World cassava production (from FAO 2000).**

*LA & C = Latin American and Caribbean regions.

## 1.3 SOME MAJOR CASSAVA DISEASES

A long growth cycle and variable climatic and edaphic conditions in the regions where the plant is cultivated, make cassava very vulnerable to attack from pathogens. In fact, over 30 different pathogens comprising a wide range of fungi, bacteria, viruses, virus-like particles, mycoplasms and arthropods, including 200 species of mites, thrips, stemborers, hornworms, whiteflies and

scale insects have been recorded (Lozano et al. 1981). Despite this, many of them cause little or no serious economic loses, but others can be considered as major pests (Bellotti & van Schoonhoven 1978). Cassava has the capacity to recover from the majority of pest attacks, if climatic conditions are favourable, especially during the rainy season (Lozano et al. 1981).

One of the bacterial diseases which is considered most important is cassava bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). *Xam* was first reported in Brazil with the name *Bacillus manihotis* (Bondar 1912). After this first report, several names were used, in particular *X. campestris* pv. *manihotis*. This pathogen was recently renamed as *X. axonopodis* pv. *manihotis* (Vauterin et al. 1995). *Xam* is a gram-negative, motile, slender rod, with a single polar flagellum. 0.76-2.69 μm x 0.32-0.49 μm, it is aerobic and fast growing (Lozano & Sequeira 1974a).

In some countries CBB causes 18-92% yield loss, and these losses are reported to be more severe during wet seasons (Persley 1979). CBB symptoms are recognised by the presence of water-soaked, angular spots, blight, partial or total wilting of the branches, gum exudate on stems or green branches, and dieback and necrosis of some vascular strands of the stems and roots **(Figure 1.7)** (Lozano 1986).

**Figure 1.7 Cassava plant with CBB systemic symptoms in Popayan, Colombia.**

Principally, rain splash, insects, and contaminated propagation stems spread *Xam*. The bacterium has an extracellular polysaccharide (EPS), which it uses to attach to the epidermal surface of the leaf. CBB penetrates the host through natural openings, stomates, or wounds, as it is unable to penetrate directly the epidermal cuticle and highly lignified tissues (Boher et al. 1996; Lozano & Sequeira 1974b). Following leaf infiltration of *Xam* through stomates, transmission electron microscopy revealed the bacterium exclusively invading xylem vessels (Cooper et al. 1995). In stems inoculated by puncture, bacteria colonised xylem vessels and the intercellular spaces of vascular bundles but no other stem tissues were invaded.

Early research on *Xam* focused on descriptions, etiology, and screening of CBB in different tropical countries (Lozano & Sequeira 1974b; Lozano & Laberry 1982; Maraite & Meyer 1975; Verdier et al. 1992). Evidence from these reports showing similarities of early leaf spot symptoms induced by *X. cassavae* pv. *manihotis* (*Xam*), and *X. cassavae* pv. *cassavae* (*Xac*). These two pathovars have different modes of action in the host and a clear separation between *Xam* and *Xac* can be achieved by restriction fragment length polymorphism analysis (Verdier et al. 1994). Daniels et al. (1993), have characterised several genes in *Xanthomonas* that are required for full pathogenicity in the interaction between this pathogen and crucifers. For example a structural gene encoding extracellular plant cell-wall degrading enzymes and genes involved in the synthesis of extracellular polysaccharides.

Restriction fragment length polymorphism (RFLP) analysis using a ribotyping technique with a 16+23S rRNA probe from *Escherichia coli* in 326 strains of *Xam* from 22 countries, showed that geographical origin determines the diversity of strains of *Xam* (Verdier et al. 1993). It was concluded that *Xam* is genetically more diverse in South America than in Africa. The results were confirmed using plasmid profiles and amplified fragment length polymorphism (AFLP) fingerprinting (Despres et al. 2000).

In different tropical countries, strategies such as crop rotation, removal and destruction of diseased plants and utilisation of clean propagative stems, have been implemented in attempts to control the disease (Lozano & Sequeira 1974a). The use of resistant cultivars is the only practical method of controlling CBB, and several resistant cultivars had been identified in different locations such as Colombia and Nigeria (Lozano & Sequeira 1974a). Traditionally,

disease-resistance varieties are developed in breeding programmes. However, there is a lack of agreement on methods of inoculation to select disease-resistance varieties and breeding methods are slow and take several years in cassava (R.M Cooper, personal communication). Cassava cultivars showing degrees of resistance to *Xam* can be identified. Ninety three varieties of *Manihot esculenta* Crantz from the cassava core collection at CIAT (Centro Internacional de Agricultura Tropical) were screened for resistance to cassava bacteria blight (CBB), and it was reported that the resistance was broadly distributed in cassava germplasm (Sánchez et al. 1999). Recently, (Restrepo et al. 2000) evaluated nineteen cassava cultivars for resistance to CBB in two edaphoclimatic zones in Colombia and selected several resistant cultivars for use in breeding programmes. (Lozano & Sequeira 1974b) suggested that CBB resistance is regulated by a multigenic response, which has yet to be characterised, and that the interaction between cassava and *Xam* does not seem to follow a simple gene for gene model. Therefore, resistant material, if identified, should be more robust, as polygenic, than would be the case in a monogenic system.

Genetic modification opens the possibility of other approaches to generating resistance to *Xam* in cassava. The *Xa21* gene from rice confers resistance against *X. oryzae* pv. *oryzae* (*Xoo*) (Century et al. 1999). Either the rice *Xa21* gene, or cassava homologues, could be transformed into cassava and their effects on CBB evaluated. Five BAC clones from cassava have been identified containing *Xa21* homologues and these BAC DNAs are to be bombarded into cassava to investigate their effect on the defence of the cassava against CBB (Nigel Taylor, personal communication).

Other important bacterial diseases are bacterial angular leaf spot caused by *X. cassavae*, bacterial stem rot caused by *Erwinia carotovora* pv. *carotovora* and bacterial stem gall produced by *Agrobacterium tumefaciens* (Bellotti & van Schoonhoven 1978).

Amongst the main diseases caused by viral and virus-like agents is frog skin disease. This disease is characterised by a reduced number of roots and thickening of root epidermis with yield loss of up to 100%, making it one of the most serious diseases of cassava. Other viral diseases are common mosaic, leaf vein mosaic, Colombian cassava mosaic virus, and African cassava mosaic virus (ACMV) **(Figure 1.8)**. By hybridising cassava with ceara rubber (*Manihot glaziovii*), and subsequent backcrossing some resistance to African cassava mosaic virus (ACMV) was developed at IITA (International Institute of Tropical Agriculture, Ibadan, Nigeria) (Ssemakula et al. 1997). By genetic engineering techniques new approaches to improve cultivars in terms of resistance against to ACMV, are being attempted. For example, transgenic cassava plants have been produced containing the coat protein gene of ACMV, viral antisense RNAs, truncated viral gene products and defective interfering DNA (DI) for the AC1 gene in ACMV. The effects of these transgenes on the resistance of cassava to mosaic virus diseases are still being assessed (Schöpke et al. 1998; Schârer-Hernández et al. 1998). A molecular linkage map, with markers linked to quantitative trait loci (QTLs) for ACMV and CBB has been developed at CIAT (Fregene et al. 1997).

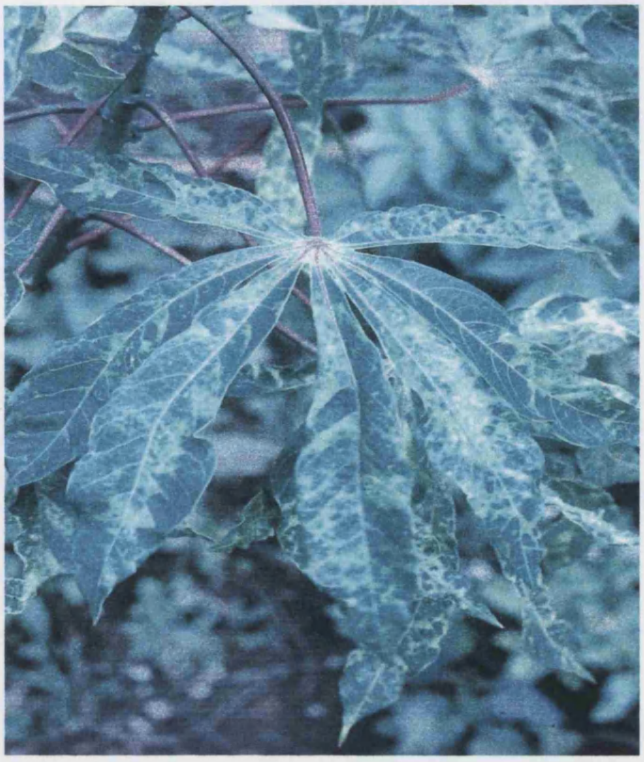

**Figure 1.8 Cassava plant affected with ACMV near Kampala, Uganda.**

One of the most common fungal diseases in cassava is produced by *Cercosporidium henningsii,* so called "brown leaf spot". Symptoms consist of uniformly distributed brown spots on both sides of the leaf, which cause total defoliation at the end of the rainy season. Leaves are attacked by many other fungal pathogens like *Cercospora vicosae*, *Phaeoramularia manihotis*, *Phoma* sp., *Oidium manihotis*, *Sphaceloma manihoticola*, *Uromyces* spp, and *Periconia shyamala*. In addition, it has been reported that cassava suffers from anthracnose caused by *Colletotrichum* spp. or *Glomerella* spp (Lozano et al. 1981). Two new diseases produced by fungi have recently been reported in West Africa, Curvularia leaf blight and Curvularia stem blight, both caused by *Curvularia lunata* Wakker, Boedijn (Msikita et al. 1997).

Since roots are so important commercially, special attention had been drawn to diseases and factors affecting this organ such as soft root rot caused by *Phytophthora drechsleri, Pythium* sp., *Fusarium solani, Fusarium oxysporum*, *Thielaviopsis basicola*, and dry root rot produced by several pathogens such as *Sellinia necatrix*, *Armillariella mellea*, *Rigidoporus lignosus*, *Diplodia manihotis*. Root smallpox caused by subterranean sucking insects, indirectly damages the root by localised lesions (Lozano et al. 1981).

Although the greatest diversity of insects attacking cassava is from the Americas, it appears that the pest complexity varies enormously over the ecological and edaphological conditions in which the plant is grown. Insect damage reduces yield by reduction of the photosynthetic area of leaves, by deficient nutrient transport following stem damage (Bellotti & van Schoonhoven 1978). Two particular species of mite, *Mononychellus tanajoa* and *Tetranychus urticae*, produce the biggest crop losses of 20 to 53% (Bellotti & van Schoonhoven 1978).

Engineering insect resistance into cassava is also being attempted, for example the *cry*1A gene from *Bacillus thuringiensis* (which confers resistance to Lepidoptera), was inserted into the T-DNA of plasmids pBIOCry and pKC2301. These plasmids were introduced into cotyledon leaves derived from somatic embryos of MCol 2215, a cassava cultivar susceptible to the stem borer *Chilomina clarkei* (lepidoptera), though the resistance is still under study (Chavarriaga et al. 1998).

Cassava germplasm collections are maintained in Brazil (CENARGEN), and Colombia (CIAT); one of their key roles it is to preserve diversity which in the future could be a source of genes from which obtain disease-resistance

cultivars against the more serious diseases of cassava (Cordeiro et al. 1994; Hershey 1987). Despite the advances mentioned above, it is still unknown what elements confer resistance in cultivars of cassava to any pathogen, as very few studies on resistance mechanisms in this crop have been attempted. In many other species, advances in understanding active/induced defence responses have come from studies of suspension cells challenged with elicitors (microbial or endogenous) of defence-related genes. This strategy will form a major part of the research described herein.

## 2 PLANT-PATHOGEN INTERACTIONS

Interactions between plants and microorganisms are highly complex, due to the involvement of two genomes (pathogen and host plant). These interactions can basically be classified into two general categories: *compatible* and *incompatible*. Compatible interactions, caused by infection of a susceptible host by a virulent pathogen, involve microbial colonisation, development of symptoms and finally disease. On the other hand, incompatible interactions between a resistant host and an avirulent pathogen are characterised by limited microbial growth, lack of symptoms and often the development of a localised necrotic lesion referred as a the hypersensitive response (HR) (Conrath et al. 1989). HR will be dealt with in more detail later. A race-specific interaction occurs when varieties of the same plant species are differentially resistant to different races of a given species of pathogen. On the other hand, race non-specific resistance is shown when a variety of a particular plant species is similarly resistant to infection by all races of a given pathogen (Walton 1997).

From his work with flax rust, Flor (1971) proposed the gene-for-gene hypothesis, which states that a gene-for-gene interaction occurs, between matching plant resistance genes (*R*), and pathogen avirulence genes (*avr*), in which dominant forms of both result in resistance but all other combinations lead to disease **(Figure 1.9)**. Several biochemical models for gene-for-gene interactions have been proposed. One is that the primary gene products of avirulence genes interact directly with the products of resistance genes (Conrath et al. 1989). Some *avr* genes have been successfully isolated from bacterial sources, where they are located on the chromosome or plasmids. For instance, the avirulence gene *avrBs3* mediates specific recognition by the pepper plant resistance gene *Bs3*, leading to the induction of the hypersensitive reaction (HR) (Bonas et al. 2000).

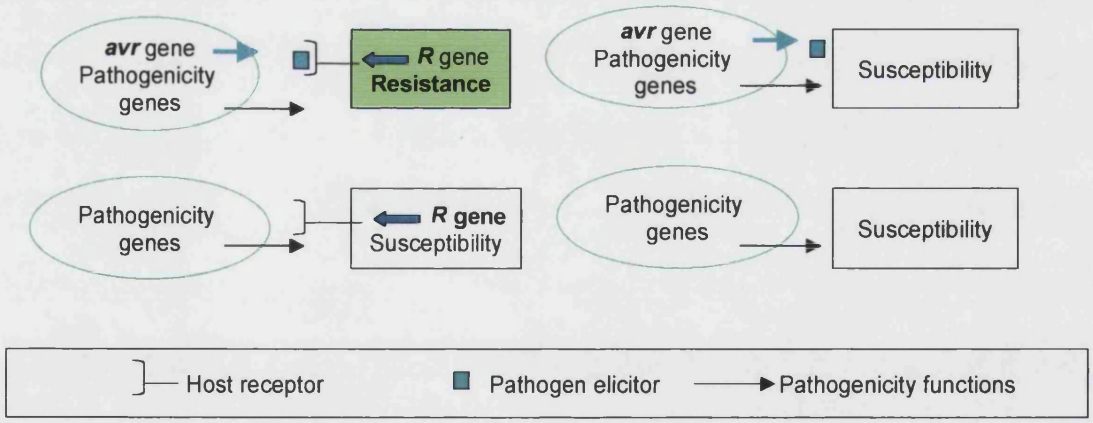


**Figure 1.9 A model based on the gene-for-gene hypothesis (after Staskawicz et al. (1995)).**

Several *R* genes have been described, and some of their functions have been identified **(Table 1.3)** (Lucas 1998). For example, *Xa21* from rice has been cloned and has been shown to confer resistance to several isolates of *X. oryzae* pv. *oryzae* (*Xoo*) in transgenic IRBB21 rice, which is normally susceptible to the pathogen (Wang et al. 1996). *Xa21* encodes a receptor-like protein kinase with leucine-rich repeats (LRRs) joined by a single transmembrane domain, suggesting a role in cell surface recognition of a pathogen ligand and subsequent activation of an intracellular kinase leading to a defence response cascade (Century et al. 1999). In tomato, the disease resistance gene, *Pto*, confers race-specific resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Hopkins 1999). *Pto* gene encodes a cytoplasmic serine/threonine protein kinase with similarities to the *Xa21* gene product (Loh et al. 1995)

| Gene | Host | Pathogen | Location | Function |
|---|---|---|---|---|
| *Pto* | Tomato | *Pseudomonas* | Cytoplasmic | Kinase |
| *RPS2* | *Arabidopsis* | *Pseudomonas* | Membrane | Receptor |
| *Xa21* | Rice | *Xanthomonas* | Membrane | Receptor |
| *N* | Tobacco | *TMV* | Cytoplasmic | Receptor |
| *Cf9* | Tomato | *Cladosporium* | Membrane | Receptor |
| *RPP5* | *Arabidopsis* | *Peronospora* | Membrane | Receptor |
| *L6* | Flax | *Melampsora* | Cytoplasmic | Receptor |
| *Hm1* | Maize | *Helminthosporium* | Cytoplasmic | Toxin reductase |

**Table 1.3 Some cloned plant disease resistance genes (*R* genes) (after Lucas (1998)).**

Other bacterial pathogenicity genes that have been studied are *hrp* genes. These genes organised in clusters and involved in plant-bacterial interactions are known as *h*ypersensitive *r*esponse and *p*athogenicity genes (***hrp***). Mutation of these genes is observed as loss of HR-inducing ability on non-host plants and severe reduction or loss of pathogenicity on hosts. Some products of genes in the *hrp* cluster can be recognised by plants, for example, the *hrpN* gene of the fireblight bacterium *Erwinia amylovora* encodes a cell-surface associated protein, known as "harpin". Harpin is a potent elicitor (a signal molecule that elicits defence responses in the plant similar to those induced by a pathogen) of HR, causing electrolyte leakage from plant cells, and finally total collapse of the plant cells (Wei et al. 1992). Moreover, harpin is considered to be responsible for triggering the production of reactive oxygen species (ROS) in tobacco suspension cells. ROS were not induced in tobacco suspension cells when *Erwinia amylovora* mutants (that do not produce harpin) were used (Baker et al. 1993). The production of reactive oxygen species in plants will be discussed in more detail.

## 2.1 PLANT DEFENCES

As a consequence of their sessile life, plants are continually exposed to the onslaught of potentially damaging situations caused by wounding, attacks by pathogens, or other stresses. These challenges trigger a complex and co-ordinated battery of defence-responses in an attempt to ward off stress-inducing agents and preserve the plant (Dixon et al. 1994; Ebel & Mithöfer 1998). Although protection by induced responses is important, it is not the only strategy

used by plants to defend themselves. The classification of plant-protection mechanisms involves both *constitutive* (passive), and *inducible* (active) elements, and a degree of interaction between both of them in terms of timing and complexity offers the plant a broad arsenal of weapons against diseases, or stresses (Lucas 1998; Smith 1996).

Before signal transduction starts, an arsenal of mechanical barriers are already present on and in plant surfaces and cell walls, and preformed compounds such as antimicrobials are present. These are the first obstacles encountered by pathogens.

The initial step in the response is the recognition of the pathogen by the plant. This first recognition event had been suggested to be through receptors in the plasma membrane of the plant (e.g. interaction with bacterial or fungal elicitors) or by cytoplasmic receptors (e.g. interaction with virus elicitors) (Hahn 1996; Kemp et al. 1999). Signal transduction events are held to co-ordinate the activation of an array of defence responses (Hammond-Kosack & Jones 1996).

Responses that appear to be consequences of elicitor perception in several of the individual systems and are possibly related to signal transduction lead to some early responses such as the activation of pre-existing protein kinases, phosphatases, and G proteins (Ebel & Mithöfer 1998; Sopory & Minshi 1998). Downstream signalling follows a depolarisation of the plasma membrane, induction of ion fluxes, the alkalinisation of the extracellular space, protein phosphorylation/dephosphorylation and the production of $H_2O_2$ (Ebel & Mithöfer 1998). These early processes appear to be mediated through the regulation of plasma membrane-bound enzymes, including changes in $Ca^{2+}$-ATPase and $H^+$-ATPase activities, and the induction of a plasma membrane-

bound NADPH oxidase or pH-dependent peroxidases (Blumwald et al. 1998; Bolwell et al. 1991; Gaudriault et al. 1997; Vera-Estrella et al. 1994).

Once the earliest events have been activated, different biochemical pathways are activated and new signal molecules are generated **(Figure 1.10)**. Plant receptor proteins (Rp) intercept signals. These signals include the direct or indirect products of avirulence (*Avr*) genes and may include the products of resistance (*R*) genes. The immediate downstream changes involve kinases, phosphatases, G proteins, and ion fluxes. Reactive oxygen species (ROS) induction, direct induction of gene transcription, or possible apoptosis genes, jasmonic acid biosynthesis, and/or ethylene synthesis may occur. Amplification of the initial response occurs through the generation of lipid peroxides, other ROS, benzoic acid, and salicylic acid. These compounds consequently induce other defence-related genes and modify defence proteins and enzymes. Simultaneously, alterations of cellular redox and/or cellular damage will activate preformed cell protection mechanisms (e.g. Halliwell-Asada cycle, superoxide dismutases, catalases, and peroxidases) and induce genes encoding various cell protectants. Defence-related stress may also induce cell death. Additionally, cross-talk between various induced pathways will co-ordinate the responses in positive (+) and negative (-) interactions (Delledonne et al. 1998; Hammond-Kosack & Jones 1996; Wojtaszek 1997).

### 2.1.1 Pre-existing Defence Structures

#### 2.1.1.1 Plant Surface Components

The plant surface presents the first barrier to entry of pathogens. The hairs on leaf and fruit surfaces exert a water-repelling effect, impeding the

attachment of some fungi and bacteria (Lucas 1998). The outer surfaces of a vascular plant are covered with a multilayered waxy deposit (network) called the cuticle, which is the first mechanical obstruction encountered by pathogens. Cutin, the structural component of cuticle, is a heterogeneous polymer of long-chain (typically 16 or 18 carbons) hydroxylated fatty acids. Esterification of hydroxyl and carboxyl groups form cross-links, which establish a polymeric network embedded in wax (Hopkins 1999). Cutin is formed as a continuous layer on the surface of external tissues such as stems, fruits, flowers and leaves (Bernards & Lewis 1998). The cuticular wax contains many classes of relatively non-polar $C_{20}$-$C_{32}$ aliphatic compounds, principally

**Figure 1.10 Speculative model of signalling events controlling activation of plant defence responses (from Hammond-Kosack & Jones (1996)). See next page.**

**ACC** oxidase, 1-aminocyclopropane-1-carboxylate oxidase; **BAG**, benzoic acid glucoside; **BA2H**, benzoic acid-2 hydroxylase; **CA**, cinnamic acid; **CHS**, chalcone synthase; **EFE**, ethylene-forming enzyme; $HO_2^{\cdot}$, hydroperoxyl radical; **HPDase**, hydroxyperoxide dehydrase; **GP**, glutathione peroxidase; **GST**, glutathione S-transferase; **k**, kinase; $O_2^{\cdot}$ superoxide anion; $OH^{\cdot}$, hydroxyl radical; **OGA** and **OGA-R**, oligalacturonide fragments and receptor; **p**, phosphatases; **PAL**, phenylalanine ammonia-lyase; **PGases**, polygalacturonases; **PGIPS**, plant polygalacturonic acid inhibitor proteins; **Phe**, phenylalanine; **PR**, pathogenesis-related genes; **Rp**, plant receptor protein; **SA** and **SAG**, salicylic acid and salicylic acid glucoside; **SA***, SA radical; and **SOD**, superoxide dismutase

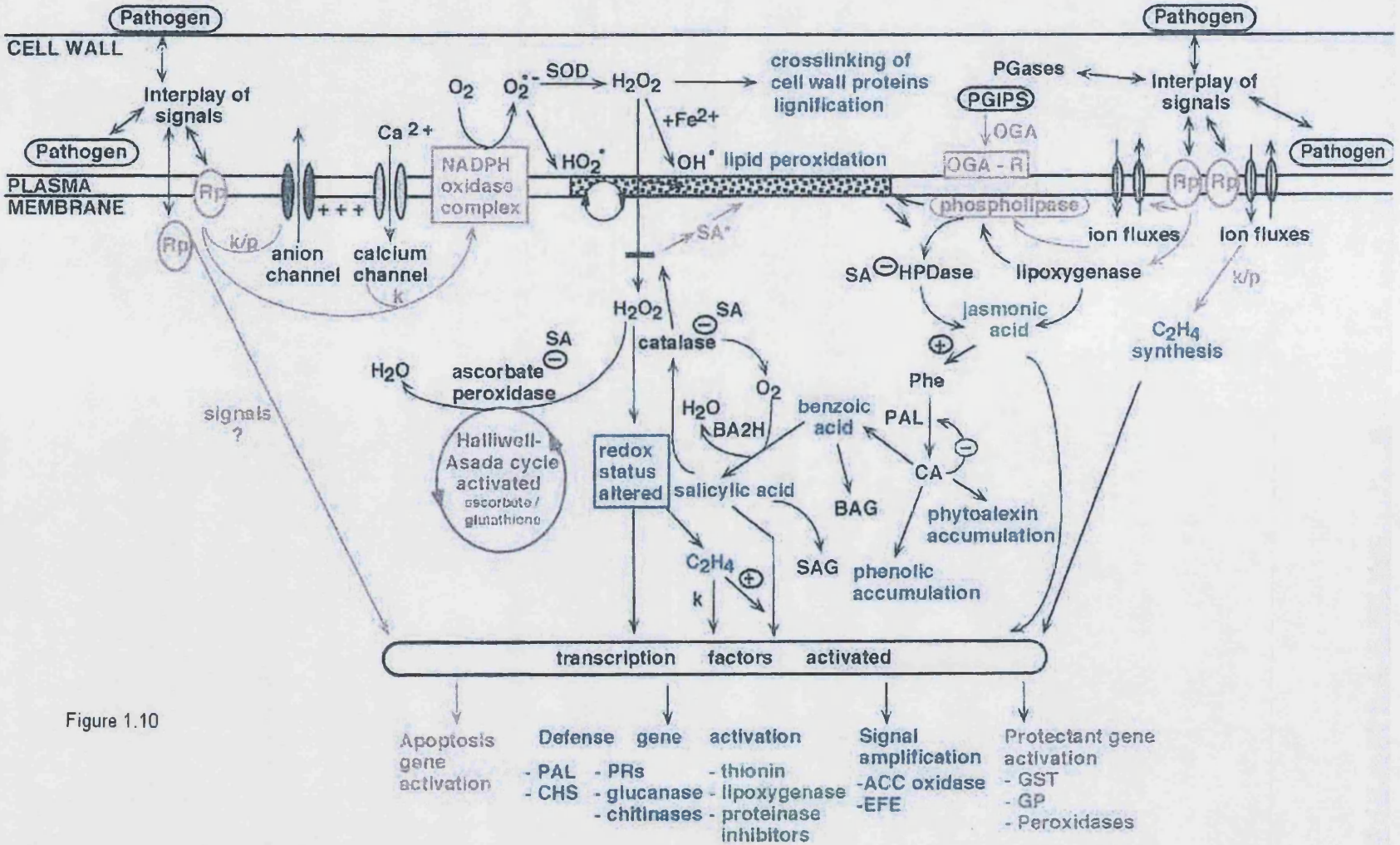
Pathogen
CELL WALL
Interplay of signals
Pathogen
PLASMA MEMBRANE
Rp
k/p
anion channel
calcium channel
Ca 2+
+ + +
k
NADPH oxidase complex
O2
O2•-
SOD
H2O2
HO2•
+Fe2+
OH•
lipid peroxidation
crosslinking of cell wall proteins lignification
PGases
PGIPS
OGA
OGA - R
phospholipase
Interplay of signals
Pathogen
Rp
ion fluxes
ion fluxes
SA•
SA
HPDase
lipoxygenase
jasmonic acid
k/p
C2H4 synthesis
H2O2
SA
catalase
H2O
ascorbate peroxidase
SA
O2
Phe
PAL
H2O
BA2H
benzoic acid
CA
signals ?
Halliwell-Asada cycle activated
ascorbate / glutathione
redox status altered
salicylic acid
BAG
phytoalexin accumulation
C2H4
k
SAG
phenolic accumulation
transcription factors activated
Apoptosis gene activation
Defense gene activation
- PAL
- CHS
- PRs
- glucanase
- chitinases
- thionin
- lipoxygenase
- proteinase inhibitors
Signal amplification
-ACC oxidase
-EFE
Protectant gene activation
- GST
- GP
- Peroxidases

Figure 1.10

hydrocarbons, such as esters, primary alcohols, and fatty acids (Brett & Waldron 1996). Thus, cuticular waxes have a dual role as a first line of protection against invaders and, due to their very high very hydrophobicity, they also confer high resistance to diffusion of both liquid water and water vapour from the underlying cells (Hopkins 1999).

The epidermal cell wall is the next potential obstruction encountered by pathogens. However, natural openings such as stomata and lenticels provide possible routes for some pathogens to overcome this initial impediment. Many pathogens have specific enzymes that can degrade plant cell walls, known as cell wall degrading enzymes (CWDE); at least 20 different cell wall degrading enzymes have been reported from plant pathogens (Walton 1994). CWDE are generally extracellular glycoproteins of low molecular weight (Cooper 1983). Virtually, all bacteria and fungi produce an array of CWDEs of every class including glycanases such as pectinases, xylanases, cellulases, and glycosidases such as, β-D-galactosidase. Various findings implicate these enzymes in infection processes *in planta*. For instance, cellulase and xylanase activity was observed in the intercellular washing fluids of bean plants after infection with bean rust *Uromyces appendiculatus*. Cellulase was detected at 2 days and reached maximum activity between 7-8 days after inoculation, while xylanase did not increase due to rust infection (Ohta et al. 2000). In *Xam* a cell wall degrading enzyme pectate lyase (PGL) was detected and this enzyme was shown to decrease the viability of cassava suspension cells. Isoelectric focusing of enzymes secreted from *Xam* grown on host cell walls revealed a single isozyme of PGL (Deshappriya 1992).

## 2.1.2 Defence Structures Formed in Response to Infection

### 2.1.2.1 Cell Wall Strengthening

Plant cell walls are highly complex and comprise a chemically and physically interlinked combination of polysaccharides, proteins, lignin, suberin, water and inorganic elements. When challenged by pathogens, wounded or stressed, plant cell walls can undergo changes in composition and structure (Showalter 1993). Among the plant cell wall components whose increase had been associated with defence response processes in plants are suberin, lignin, hydroxyproline-rich glycoproteins (HRGPs), callose, and enzyme cell wall proteins such as peroxidases. (Bach & Seitz 1997; Faulkner & Kimmins 1975; Grosskopf et al. 1991; Showalter et al. 1985).

Despite the importance of suberisation during normal growth, developmental processes, and as a stress response in plants, its nature is only now becoming clear. Suberised tissues contain polyaromatic and polyaliphatic domains. The aromatic domain is composed of (poly)-hydroxycinnamates, such as amides (e.g., feruloyltyramine). On the other hand, the aliphatic domain presents a multi-lamellar structure, which contains chains of fatty acids (>20 carbon units), with C-16 and/or C-18 alkan-$\alpha,\omega$-diodic acids (Bernards & Lewis 1998). Suberisation of layers of cells at the surface of the wounds protect the plant. The cork layers possibly serve to block the spread of toxic substances that the pathogen may secrete, and to prevent the flow of nutrients and water from the healthy to the infected area, so as to isolate the pathogen, and deprived it of nutrients. Several enzymes involved in suberisation increase in activity under elicitor challenge in bean (*Phaseolus vulgaris* L.) suspension cells (Bolwell et al. 1997), and suberin content increased in plant cells bordering

mechanical wounding in leaves (Faulkner & Kimmins 1975). Another tactic found in plants is the formation of abscission layers, which generate gaps between two circular layers of leaf cells surrounding the locus of infection. Eventually, this area dies, and is discarded from the plant. Tyloses are other structures that interfere with the spread of vascular pathogens. They are derived from the expansion of protoplasts of adjacent living parenchymatous cells, which results in occlusion of xylem vessels (Lindgren 1997). For example in cassava tyloses appear in the stele of wounded plants. Despite the presence of tyloses in both resistant and susceptible cultivars of cassava against cassava bacterial blight, some tyloses show a specific differentiation associated with the secretion of phenolic compounds which may be bactericidal and slow down bacterial multiplication (Boher et al. 1996).

Similarly, gum accumulates in xylem vessels, in intercellular spaces and within the cells surrounding the point of infection, thus forming a barrier that encloses the pathogen. Tyloses and gums are strengthened by the infusion, and polymerisation of phenolic compounds, which inhibit fungal enzymes and form a permanent seal around the infected zone in angiosperm trees (Ellis et al. 1997). For example, terpenoid aldehyde phytoalexins formation occurred simultaneously with the formation of tyloses, in cotton plants inoculated with *Verticillium albo-atrum* (Kolattukudy & Köller 1983)

Lignin is an integral cell-wall constituent of all vascular plants and woody tissues, and is considered a key component in defence response processes. When lignin monomers polymerise they form covalent cross-links with polysaccharides and proteins. This event contributes to strengthening the cell walls against mechanical disruption, increasing resistance to degradation by

microbial attack, and increasing water impermeability of the polysaccharide protein matrix of the cell wall (Boudet 1998; Whetten et al. 1998). In *Populus tremuloides* wound-inoculated with *Entoleuca mammata,* a resistant genotype showed substantially higher PAL and CAD activities than a susceptible and their were differences in lignin monomeric composition between the two genotypes (Bucciarelli et al. 1998). This supports the roles of phenylpropanoid enzymes and lignin biosynthesis in plant defence. Developmentally, depositions of lignin are often associated with cells and tissues that have ceased to grow and are involved in mechanical support (e.g. sclerenchyma), conduction (e.g. xylem), or protection and defence (e.g. periderm) (Boudet 1998).

Lignin is formed through the oxidative polymerisation of cinnamyl alcohols (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) **(Figure. 1.11)**. The relative proportion of these alcohol monomers varies between plant species, between cell types within a single plant, and between different parts of the wall of single cells, and also in response to the environment (Boudet 1998). Among gymnosperms lignin is composed principally of guaiacyl subunits (G units), and a small proportion of ρ-hydroxyphenyl units (H units). By contrast in angiosperms lignin is composed of syringyl units (S units), G units, and H units (Whetten et al. 1998). Mutant and transgene analysis has revealed several unusual lignin units, which lead to a reconsideration of the complexity and flexibility of the lignin biosynthesis and polymerisation in plants **(Table 1.4)**. For example, a loblolly pine *Pinus taeda* mutant depleted in cinnamyl alcohol dehydrogenase (CAD) activity, known as *cad-n1*, showed normal development under glasshouse conditions but presented a novel lignin content with very different structural characteristics. This novel lignin accumulated free

coniferaldehyde to high levels and had a low accumulation of *p*-coumarylaldehyde, although levels of *p*-hydroxyphenyl units were unchanged (Ralph et al. 1998). So far it is not known whether this novel lignin confers altered responses to pathogens or elicitors.

Lignification processes occur in plant suspension cells challenged by various elicitors. For instance, larch suspension cells, treated for five days with a cell wall glucan elicitor derived from *Fusarium oxysporum* Schlecht, exhibited a four to six-fold increase of cell wall bound lignin (Bach & Seitz 1997). Accumulation of lignin reached a 10-fold increase in castor bean (*Ricinus communis*) suspension cultures challenged with a pectic fragment elicitor within three hours of elicitation, and peaked between 4-10 hours after elicitation (Bruce & West 1989).

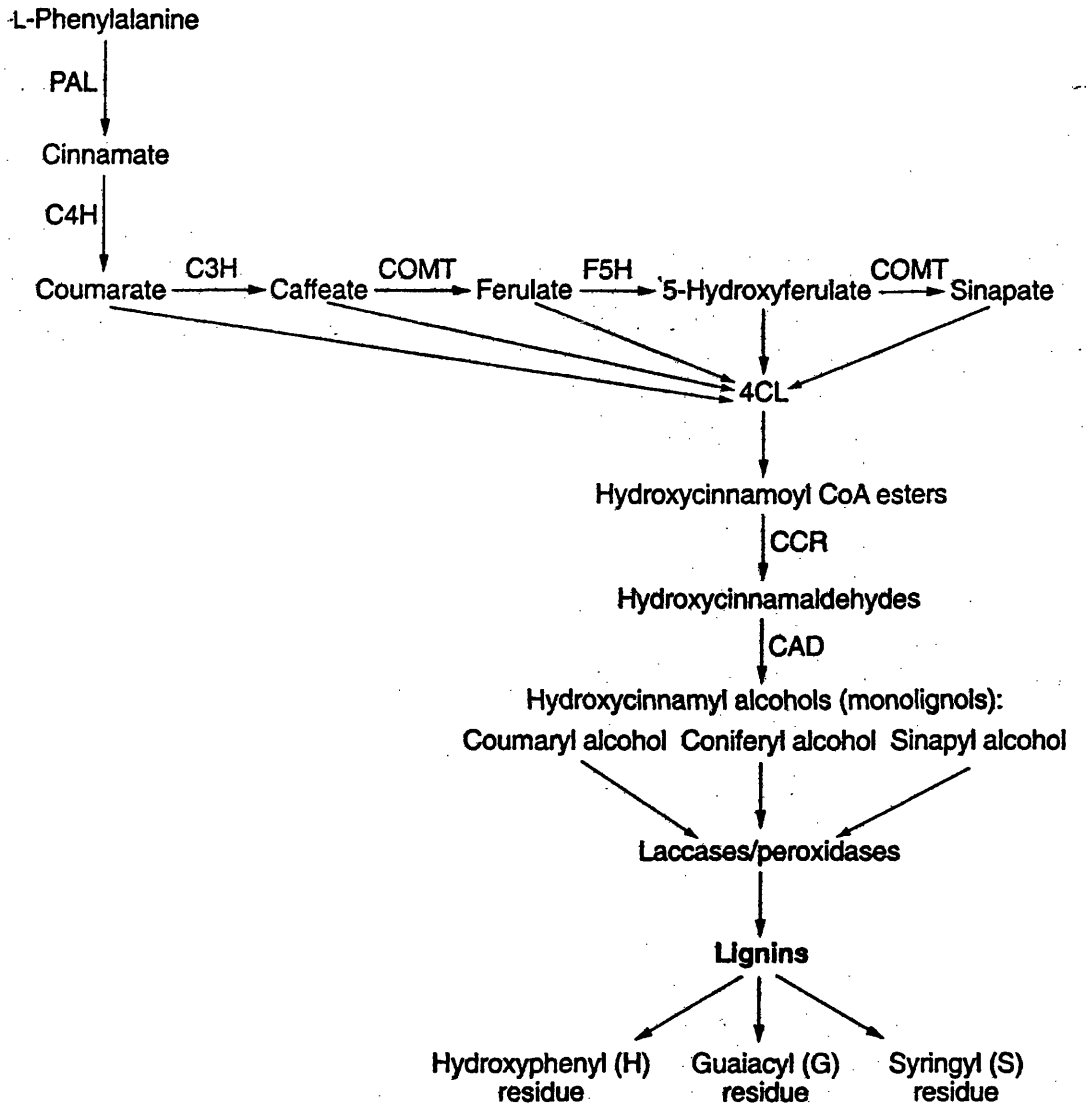


**Figure 1.11 Lignin biosynthesis pathway (from Boudet (1998))**

**PAL**: Phenylalanine ammonia-lyase; **C4H**: Cinnamate 4-hydroxylase; **C3H**: 4-hydroxycinnamate 3-hydroxylase; **COMT**: S-adenosyl-methionine: caffeate/5-hydroxyferulate-O-methyltrasferase; **F5H**: Ferulate 5-hydroxylase; **4CL:** Hydroxycinnamate: CoA-ligase; **CCR**: Hydroxycinnamoyl-CoA: NADPH oxidoreductase; **CAD:** Hydroxycinnamyl alcohol dehydrogenase.

| Transgenic method | Species | Enzyme effect[1] | Lignin content[2] | Lignin composition[3] | Reference |
|---|---|---|---|---|---|
| Antisense *cad* | Poplar | CAD 30-50% | No effect | Increased aldehyde | (Baucher et al. 1996) |
| Overexpression *pod* | Tobacco | POD increased | Increased to ~ 130% | n.d. slower growth | (Lagrimini 1991) |
| Sense suppression of *pal* | Tobacco | PAL decreased | Decreased | Increased S:G | (Sewalt et al. 1997) |
| Introduction of *tdc* | Potato | TDC active | Decreased 60% | Decreased S:G | (Yao et al. 1995) |

**Table 1.4 Selected examples of modification of lignin in transgenic plants (after Whetten et al. (1998)).**

[1]Enzyme effect: level of enzyme activity relative to wild type (%); [2]Effect on lignin content, % of wild type content; [3]Effect on lignin composition relative to wild type; **CAD**, cinnamyl alcohol dehydrogenase; **n.d.**, not determined; **PAL**, phenylalanine ammonia-lyase; **POD**, peroxidase; ***tdc***, tryptophan decarboxylase.

A particular group of glycosylated proteins known as hydroxyproline-rich glycoproteins (HRGPs) are important components of plant cell walls and are widely distributed in the plant kingdom. HRGP is a generic name that covers all molecules rich in hydroxyproline (Hyp) and proline (Pro) in the protein backbone, with a pentapeptide repeating sequence Ser-$Hyp_4$, and a glycosyl component which contains arabinose and galactose as the major monosaccharides (Corbin et al. 1987; Sommer-Knudsen et al. 1998). Additionally, some HRGPs are rich in other amino acids such as serine (Ser), valine (Val), tyrosine (Tyr), and lysine (Lys), and/or have different repeat sequences (Showalter et al. 1985). HRGPs have been classified into four main

subgroups; extensins, arabinogalactan proteins (AGPs), proline-rich proteins (PrPs) (known as P/HRGPs), and solanaceous lectins (Sommer-Knudsen et al. 1998). Additionally, glycine-rich proteins (GRPs) are another subgroup that falls in this basic classification of HRGPs (Showalter et al. 1985).

Functionally, HRGPs are involved in the control of cell wall structure during developmental processes, and in strengthening plant cell walls by the formation of peroxidase-mediated intermolecular cross-links in defence responses (Cooper & Varner 1983; Cooper & Varner 1984; Ye et al. 1991). By using an inhibitor of prolyl hydroxylase, 3,4-dehydro-L-proline (Dhp), on tobacco protoplasts (Cooper et al. 1994) concluded that HRGPs are crucial for cell wall structure and necessary for proper cell division and morphology in tobacco cells. HRGPs may function in defence as specific agglutinins of microbial pathogens, and by directly forming structural barriers or by providing sites for lignin deposition (Leach et al. 1982). Accumulation of HRGPs has been associated with the expression of disease resistance against different pathogens, stresses, and elicitors **(Table 1.5)** (Bradley et al. 1992; Showalter et al. 1985). For instance, the accumulation of HRGPs mRNAs was reported in bean suspension cells after challenging with the high molecular weight fraction released by heat treatment of isolated mycelial cell wall of *Colletotrichum lindemuthianum* and in interactions between bean hypocotyls and the living fungus (Showalter et al. 1985). HRGP and peroxidase genes were activated in fungus-infected parsley leaves and elicitor challenged parsley suspension cells, and *in situ* hybridisation demonstrated the presence of their mRNAs in parsley tissue around fungal infection sites, thereby confirming the modification of cell walls as a component of plant defence (Kawalleck et al. 1995).

| Protein Class | Condition(s) |
|---|---|
| "Extensins" (dicotyledons) | Wounding, fungal infection, viral infection, fungal elicitors, endogenous elicitors, ethylene, red light, heat shock, gravity, glutathione, cell culturing, development |
| "Extensins" (monocotyledons) | Development, wounding |
| "Extensins" (*Chlamydomonas*) | Development |
| "Extensins" (*Volvox*) | Development |
| GRPs (dicotyledons) | Development, viral infection, salicylic acid, abscisic acid, drought stress, wounding |
| GRPs (monocotyledons) | Development, water stress, abscisic acid, mercuric chloride, wounding |
| PRPs (known as P/HRGP) | Wounding, endogenous elicitors, fungal elicitor, ethylene, cell culturing, light, red light, development |
| PRPs (nodulins) | Development |
| Solanaceous lectins | Wounding, viral infection |
| AGPs | Development, wounding |

**Table 1.5 Conditions regulating the expression of HRGPs (after Showalter (1993)).**

## 2.1.3 Pre-existing Biochemical Defences

### 2.1.3.1 Antimicrobial Compounds

Among the diverse range of plant-defences there exists a range of compounds which are present in healthy plants (antimicrobial compounds), and others that can be induced *de novo* (phytoalexins) in plants that have been challenged with pathogens, elicitors, or other stresses (Lucas 1998).

Constitutive low molecular weight antimicrobial compounds, known as "phytoanticipins", are the first chemical challenges to potential pathogens. Their presence may confer non-host or race non-specific resistance. They are generally considered to be effective following penetration, rather than by inhibiting microbial development at the plant surface. A large number of constitutive plant compounds have antifungal activity. These include, phenols and phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, glucosinolates, cyanogenic glycosides, 5-alkylated resorcinols, and dienes (Osbourn 1996). Some phytoanticipins are released from inactive precursors following tissue damage and others are constitutive, being present in their biological active form. A typical example is the release of toxic cyanide from cyanogenic glycosides (Mansfield 1983; Vanetten et al. 1994). Cyanogenic glycosides are present in over 800 plant species, including cassava. Cassava cyanogenic glycosides will be dealt later. Other widely distributed antimicrobial compounds are the saponins, which are steroidal, or triterpenoid glycosides. The antifungal properties of saponins have been attributed to their capacity to complex with membrane sterols, causing pore formation and leakage of cell contents (Bangham & Horne, 1962). Some fungi produce specific saponin-detoxifying enzymes that remove sugar from the glycosyl chain at the C-3

carbon position to give products less toxic to fungal growth. Examples are, avenacinase, which is produced by the oat pathogen *Gaeumannomyces graminis* var. *avenae* (*Gga*), that detoxifies avenacin A-1 (triterpenoid) and the tomatinase enzyme, produced by the pathogen *Septoria lycopersici*, which deglucosylates the tomato saponin α-tomatine (steroidal glycoalkaloid) (Tonukari et al. 2000). Two approaches have been utilised to test the importance of saponins in plant defence, including the generation of mutants of phytopathogenic fungi defective in saponin detoxification, and the isolation of plant mutants lacking saponins (Osbourn et al. 2000). For instance, ten saponin-deficient mutants (*sad*) of the diploid oat species *Avena strigosa* had been isolated and challenged with the avenacin-sensitive fungus, *G. graminis*. While the wild type *A. strigosa* line normally fails to give disease symptoms when it is inoculated with this fungus, all ten mutants were susceptible to this fungus and developed lesions on their roots (Osbourn et al. 2000)

The distribution of these preformed inhibitors within the plant is often tissue specific, with a tendency to accumulate in the outer layers of plant organs, where they are often found in the vacuoles of healthy plants (Bennett & Wallsgrove 1994). For example, avenacosides, a saponin, is only produced in oat leaves (Tonukari et al. 2000). The nature and level of constitutive antimicrobial compounds depend on different factors such as host genotype, age, and environmental conditions (Osbourn 1996).

## 2.1.4 Early Responses

### 2.1.4.1 Ion Fluxes

The identification of the earliest responses to pathogen invasion has been a focus of attention for several years. The earliest detectable cellular events are ion fluxes across the plasma membrane and a burst of oxygen metabolism, known as the 'oxidative burst' (McDowell & Dangl 2000). Responses triggered by elicitor perception in several systems and possibly related to signal transduction include depolarisation of the plasma membrane, inducible ion fluxes, the alkalinisation of the extracellular space, and protein phosphorylation (Ebel & Mithöfer 1998).

A prerequisite for the initiation of an efficient defence response is the recognition of the potential "invader" or "stress". This is believed to be through the identification of the specific pathogen or elicitor by receptors in the plasma membrane or in the cytosol (Bonas & Van den Ackerveken 1997; Nürnberger et al. 1994; Templeton & Lamb 1988). Such specific binding receptors have been shown to exist for oligosaccharide, glycoprotein, and peptide elicitors (Hahn 1996). Several receptor genes have also been characterised (Hammond-Kosack & Jones 1996), and two of them, (*Pto* and *Xa21*), have sequence domains similar to those of protein kinases (Murphy et al. 1999).

In the signal transduction cascade, the first response is believed to include an influx of $Ca^{2+}$ (Schwacke & Hager 1992), a drop in the membrane potential, efflux of $K^+$ together with $Cl^-$ and/or $HCO^{3-}$ (as a counter ion for $H^+$ influx) (Murphy 1988; Schwacke & Hager 1992), acidification of the cytoplasm (Murphy et al. 1983), and alkalinisation of the apoplast (Bolwell et al. 1995). Changed ion fluxes result in membrane polarity changes, increases in cytosolic

$Ca^{2+}$ and $H^+$ levels, and activation of phospholipases, phosphatases and protein kinases (Hardie 1999). $K^+$ efflux also is a key element of the signal transduction pathway, since treatments that inhibited the UV-induced efflux of $K^+$ in cultured rose cell suspensions repressed the appearance of $H_2O_2$ (Murphy & Huerta 1990). External $Ca^{2+}$ and $Ca^{2+}$ channels in spruce cells elicited with fungal elicitor, and in tobacco cell suspension cells elicited by cryptogein, were essential for the oxidative burst to take place (Pugin et al. 1997; Schwacke & Hager 1992). $Ca^{2+}$ fluxes are necessary but not sufficient for the initiation of down-stream reactions in parsley cells challenged with crude cell wall preparations from the mycelium of *Phytophthora sojae* race 1 (Jabs et al. 1997). By patch-clamp analysis of elicitor-treated parsley protoplasts, Zimmermann et al. (1997) identified a specific ion channel that was transiently activated by elicitor to mediate $Ca^{2+}$ influx under physiological conditions. Using piperazines, molecules that inhibit Ca2+ fluxes, and phytoalexin accumulation in parsley cells, the involvement of distinct types of $Ca^{2+}$ channels in elicitor signal transduction in different plants was suggested (Nürnberger et al. 1994).

Elicitor induced $K^+$ efflux has been found to be associated with an extracellular alkalinization, indicating that effluxes of $K^+$ and influxes of protons, or 'proton' equivalents' are somehow coupled (Mathieu et al. 1994). Various hypotheses have been proposed to account for the extracellular alkalinization **(Figure 1.12)**. A $K^+/H^+$ antiport could catalyse coupled K+ and H+ fluxes with a stoichiometry of 1. Electrical coupling between $K^+$ channels, which could open as the result of elicitor induced membrane depolarisation, and the proton channel responsible for the entry of $H^+$ would also account for the observed fluxes of equivalent intensities. A more complex coupling, between the proton

pump ATPase, proton short circuits corresponding to $H^+$/solute co-transport and $K^+$ channels, would also account for associated proton influx and $K^+$ efflux resulting from the inhibition of the pump. Finally, an elicitor-induced efflux of bicarbonate ions associated with the $K^+$ release would leave an excess of protons in the cytosol and would trap the protons in the external medium to form carbonic acid, which is then released in the atmosphere as $CO_2$.

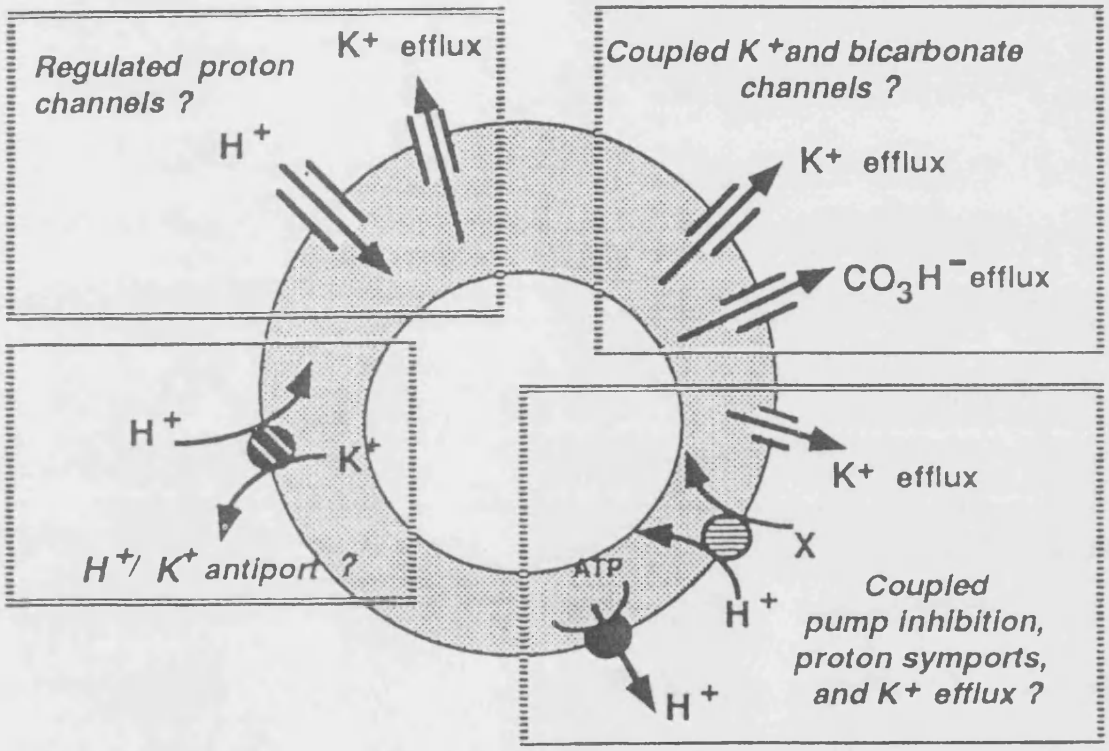


**Figure 1.12 Mechanisms for extra-cellular change of pH (from Mathieu et al. (1994)).**

Plant suspension cells have been extensively used in attempts to elucidate different steps and strategies of the defence mechanisms adopted by plant, including the changes of pH, which are linked to the hypersensitive

response and the oxidative burst. For example, in parsley cells all ion fluxes were shown to be prerequisites for the induction of the oxidative burst, but not vice-versa (Jabs et al. 1997). It was suggested that there is a feedback regulation of the oxidative burst on those ion fluxes (Cazalé et al. 1998) **(Figure 1.13).**

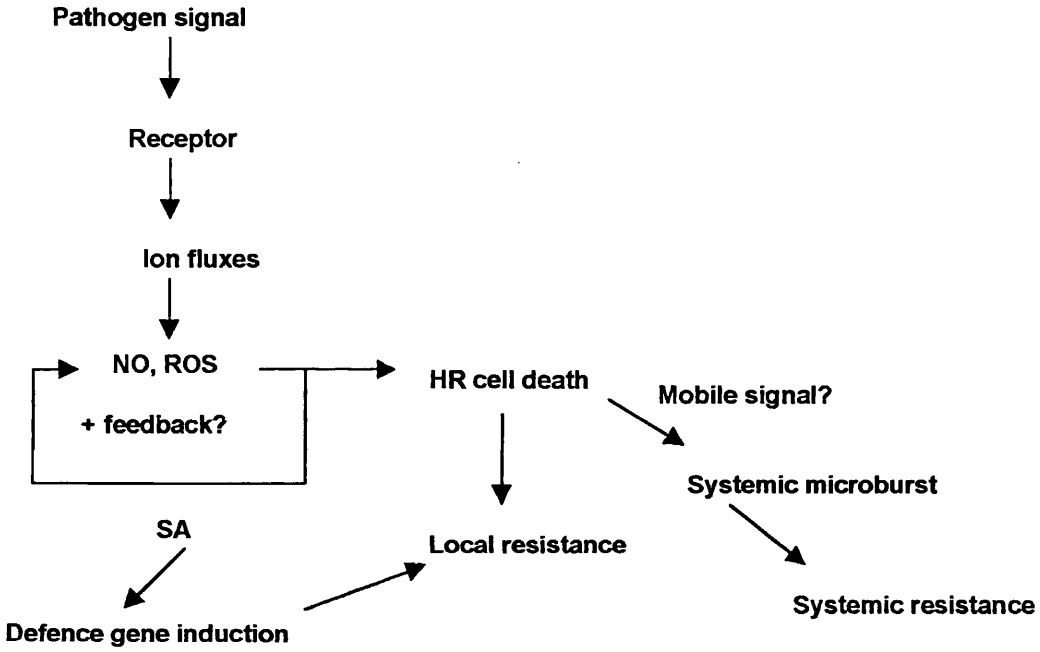


**Figure 1.13 Regulation of local and systemic defence responses (after McDowell & Dangl (2000)).**

**NO**, Nitric oxide; **ROS**; Reactive oxygen species.

#### 2.1.4.2 Oxidative Burst

Plant cells under circumstances such as attacks from viral, bacterial, or fungal pathogens, when challenged by elicitor molecules derived from pathogens, or subjected to other stresses, undergo an oxidative burst state. This is a rapid synthesis of reactive oxygen species (ROS), superficially similar to that produced by mammalian phagocytes (Low & Schroeder 1996). While, oxygen is essential for the life of aerobic organisms, these reactive oxygen species can be toxic if they are permitted to accumulate (Baker & Orlandi 1995). Ozone, singlet oxygen, hydroxyl radical, and organic oxyradicals damage plant organs, organelles, cell membranes, proteins, and DNA (Larson 1995). Oxygen toxicity can be due either to uncontrolled reactive oxygen species production, or to an inefficient system of scavenging them (Monk et al. 1989). ROS can oxidise membrane fatty acids and disrupt cellular integrity (Liese & Weiner 1996). Oxidation of amino acids is often associated with loss of biological activity, for example due to the oxidation of methionine to form methionine sulfoxide (Kpemoua et al. 1996). Other amino acids, which are also susceptible to damage by superoxide radical ($^{\bullet}O_2^-$), are tryptophan, histidine and cysteine (Iida et al. 1995). Furthermore, oxidative changes in the active centres of metalloenzymes reduce their activity. For example, superoxide can inactivate catalase by converting the ferric enzyme to the less active ferro-oxy form (Fridovich 1986). Particularly, those enzymes that contain reduced thiol (-SH) groups are readily oxidised and as a consequence lose their activity (Li et al. 2000). Hydroxyl radicals can attack all components of DNA (sugar and base), leading to a large number of DNA lesions and alterations, which can include base loss and strand breakage (Matheron & Benbadis 1994).

Control of these oxidative elements and protection of the cell from ROS in eukaryotes occurs *via* processes involving the transcription of genes that encode antioxidant enzymes (e.g. catalase, superoxide dismutase, and peroxidases). Moreover, compounds such as α-tocopherol, ascorbic acid, glutathione, β-carotene, flavonols (e.g. quercetin), flavanols (e.g. catechin), flavones (e.g. rutin) and hydroxycinnamates such as ferulic acid, have antioxidant properties (Baker & Orlandi 1995; Larson 1995; Rice-Evans- C A et al. 1997). In addition to stress-induced ROS, free radical production also takes place under normal conditions where no external stimulation has occurred. However, these are at such levels that they are capable of modification by the detoxification systems represented by catalase, superoxide dismutase (SOD) and peroxidases, which are universal across the kingdoms (Bolwell et al. 1995). In plants, the antioxidative systems are located in all subcellular compartments including the apoplastic space **(Figure 1.14)** (Polle 1997).

As ROS can be lethal to aerobic organisms, several antioxidant processes are necessary to protect plants against the accumulation of ROS. However, ROS are essential to aerobic life. The transfer of electrons from NADH and $FADH_2$ to groundstate oxygen during the oxidative phosphorylation process releases chemical energy as ATP. Furthermore, not all reactive oxygen species are harmful to the cell in all instances, for example, respiration itself results in two kinds of reactive oxygen species, superoxide radical ($^{\bullet}O_2^-$) and hydrogen peroxide ($H_2O_2$), which are unreactive on their own (Li et al. 2000).

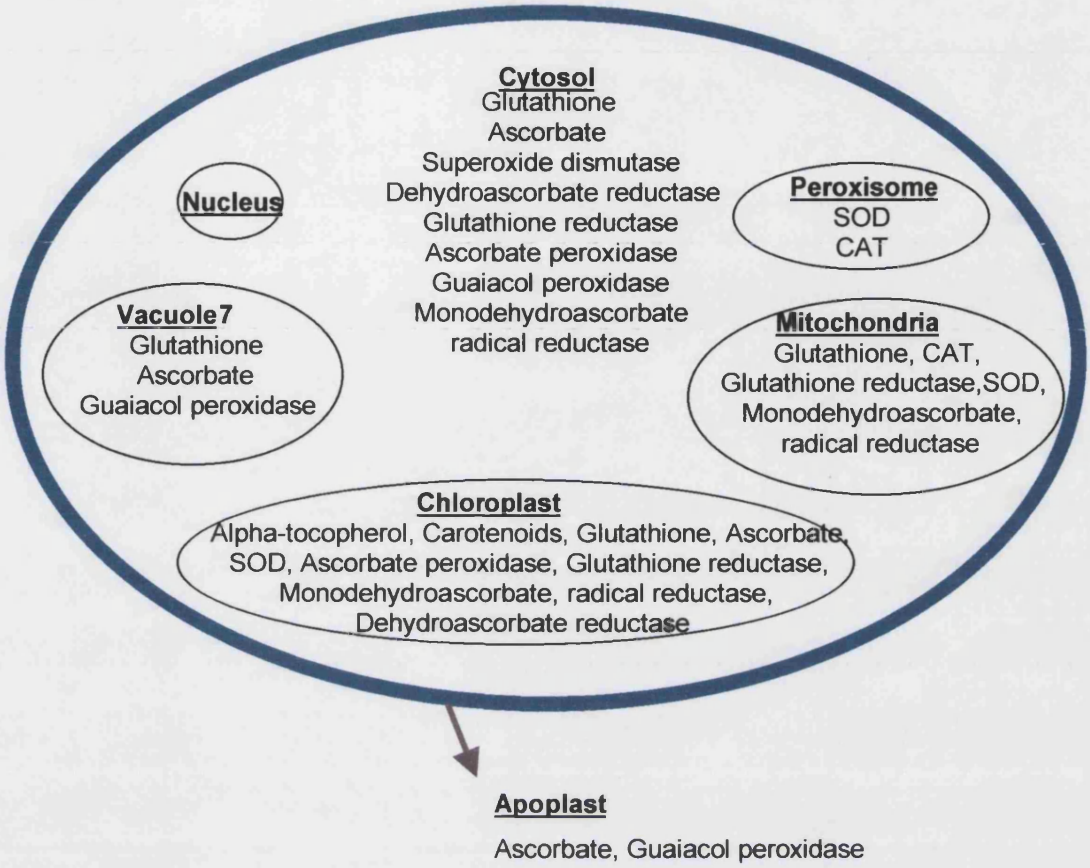


**Figure 1.14 Subcellular localisation of antioxidative systems in plant cells (after Polle (1997)).**

**SOD**: Superoxide dismutase; **CAT**: Catalase.

The production of ROS often plays a crucial role in plant defence responses. For example, hydrogen peroxide ($H_2O_2$) is involved directly in the reduction of pathogen viability, even at low concentrations (5-20 μM), and is able to inhibit the germination of spores of pathogenic fungi (Peng & Kuc 1992). Furthermore, the liberation of $H_2O_2$ permits cell-wall reinforcement by increased lignification, and cross-linking of cell wall proteins (e.g. HRGPs), thus building an impediment to the entry of pathogens (Bradley et al. 1992; Brisson et al.

1994; Oufattole et al. 2000; Wu et al. 1997). $H_2O_2$ is essential for the initiation of the hypersensitive response (HR) (Dale et al. 1989; Doke 1983; Greenberg et al. 1994; Levine et al. 1994; Messner & Meinrad 1994; Tenhaken et al. 1995), although other factors may also be needed (Glazener et al. 1996; Jabs et al. 1997). A major alteration of membranes seems to take place in the initial phases of HR. The mechanism responsible for these alterations involves a transient production of ROS (Messner & Meinrad 1994). The earliest detectable changes in HR are electrolyte leakage, cell membrane depolarisation and lipid peroxidation. Peroxidation of membrane lipids leading to membrane dysfunction together with the production of lipid-derived signals may be involved in intracellular signalling (Bolwell et al. 1995). Therefore, $H_2O_2$ (Levine et al. 1994; Wu et al. 1995), superoxide (Jabs et al. 1997) and possibly hydroxyl radicals (Mehdy et al. 1996) may serve as extracellular signals. These signal molecules can activate plant defence mechanisms, such as the induction of phytoalexin production and the expression of pathogenesis-related genes (PR proteins) (Apostol et al. 1989; Godiard et al. 1994; Green & Fluhr 1995; Jabs et al. 1997). However, others have proposed that the oxidative burst is not always a primary signal for phytoalexin biosynthesis (Davis et al. 1993). While the oxidative burst plays a major role in defensive responses, there is also evidence that suggests that it can play an important part in developmental regulation (Cazalé et al. 1998; Legendre et al. 1993). Transgenic tobacco plants that constitutively produce elevated sublethal levels of $H_2O_2$ activate host defence mechanisms such as the induction of defence-related proteins, and the peroxidase-mediated lignification of the cell wall (Wu et al. 1997).

The first report of rapid ROS production appeared in 1983, which described the generation of $^{\bullet}O_2^-$ by potato tuber discs in response to inoculation with an incompatible race of *Phytophthora infestans* or with hyphal wall components of that race, but not with a compatible race (Doke 1983) **(Table 1.6)**. Since then, several groups have reported detection of ROS *in planta*. For instance, increased ROS was observed in tomato plants, which carry the *Cf5* gene in response to the elicitor Avr9 of *C. fulvum*. The maximum increase was 20 mM of $H_2O_2$, which was detected one to two hours after inoculation and continued for at least four hours (Lu & Higgins 1998). Production of reactive oxygen species, occurs early (even within seconds or minutes) in the interactions between plant cell suspension cultures and bacteria, fungi or elicitors (Apostol et al. 1989; Auh & Murphy 1995; Baker 1991; Glazener et al. 1991). An oxidative burst was stimulated in cultured rose cells by UV-C radiation (Murphy & Huerta 1990). In some suspension cell/elicitor combinations two oxidative bursts are produced, one early and one later (Lamb & Dixon 1997; Messner & Meinrad 1994). Evidence suggests that the later burst occurs only in response to specific pathogen/receptor combinations. That is, the later burst requires the presence of "incompatible" pathogens (Baker 1991; Lamb & Dixon 1997) **(Figure 1.15)**. In some, but not all the cases, the later burst is a sign that the plant cells undergo programmed cell death or "apoptosis" (Wang et al. 1996). The early burst, in contrast, is relatively non-specific, and stimulated by both compatible and incompatible pathogens, and by extracts containing elicitors. Mechanical signals derived from degradation, puncture or deformation of the cell wall/plasma membrane or simply shaking the cells were also capable of triggering the early oxidative burst (Legendre et al.

1993; Yahraus et al. 1995). Mechanical stress or hypoosmotic stress of tobacco suspension cells activated ROS, cross-linked wall proteins and induced the activation of MAP kinases (Cazalé et al. 1998; Cazalé et al. 1999).

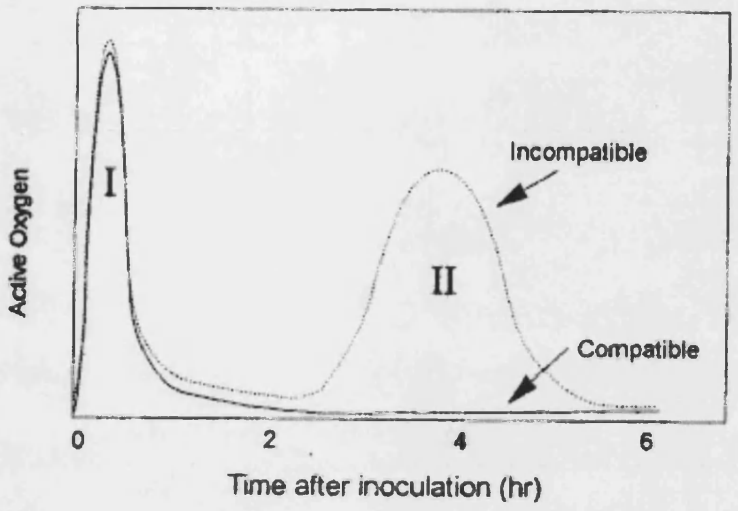


**Figure 1.15 Phases of active oxygen production in plant-bacterial interactions (from Baker & Orlandi (1995)).**

| Plant | Pathogen/type elicitor | ROS | References |
|---|---|---|---|
| **Plant-fungus interactions** | | | |
| Potato tuber discs | *Phytophthora infestans* zoospores or hyphal wall components | $^{\bullet}O_2^-$ | Doke (1983) |
| Potato protoplasts | *Phytophthora infestans* hyphal wall components | $^{\bullet}O_2^-$ | Doke (1983) |
| Soybean suspension cells | *Phytophthora megasperma* f.sp. *glycinea* cell wall preparations | $^{\bullet}O_2^-$, $H_2O_2$ | Lindner et al. (1988) |
| French bean suspension cells | *Colletotrichum lindemuthianum* crude cell-wall preparations; chitin or chitosan oligomers | $H_2O_2$ | Bolwell et al. (1995) Wojtaszek et al. (1995) |
| Tobacco suspension cells | Pure cryptogein and capsicein protein elicitors | $H_2O_2$, $^{\bullet}O_2^-$? | Rustérucci et al. (1996) |
| Rice suspension cells | *N*-Acetylchito-oligosaccharides | $H_2O_2$, $^{\bullet}O_2^-$, $^{\bullet}OH$ | Kuchitsu et al. (1995) |
| Rose suspension cells | *Phytophthora* sp. crude cell-wall preparation | $H_2O_2$, $^{\bullet}O_2^-$ | Auh & Murphy (1995) |
| **Plant-bacterium interactions** | | | |
| Soybean suspension cells | *Pseudomonas syringae* pathovar *glycinea* | $H_2O_2$ | Levine et al. (1994) |
| *Arabidopsis thaliana* suspension cells | Protein elicitor harpin | $H_2O_2$, $^{\bullet}O_2^-$ | Desikan et al. (1996) |
| **Plant-bacterium interactions** | | | |
| Tobacco leaf discs | Tobacco mosaic virus (TMV) | $^{\bullet}O_2^-$ | Doke & Ohashi (1988) |
| **Plant-plant cell-wall fragments** | | | |
| Soybean suspension cells | Oligogalacturonide | $H_2O_2$ | Levine et al. (1994) |
| **Mechanical stress** | | | |
| Soybean suspension cells | | $H_2O_2$ | Yahraus et al. (1995) |

**Table 1.6 Selected examples of plant-systems generating ROS when challenged with various pathogens/elicitors or in response to mechanical stress (after Wojtaszek (1997)).**

Strictly, ROS represent the products obtained by the successive single-electron reduction of molecular oxygen **(Figure 1.16).** ROS are normally generated at low levels by plant cells in chloroplasts, mitochondria and cytoplasm, generally by enzymes involved in reduction-oxidation processes (Baker & Orlandi 1995; Mehdy 1994) **(Figure 1.17).** These successive single-electron reductions of molecular oxygen start with a slight input of energy in the first one-electron reduction and then the cascade produce the superoxide radical ($^{\bullet}O_2^-$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical ($^{\bullet}OH$). These are the species principally detected in plant-pathogen interactions (Baker 1991; Wojtaszek 1997).

$$O_2 \xrightarrow{e^-} {}^{\bullet}O_2^- \xrightarrow[2H^+]{e^-} H_2O_2 \xrightarrow[H^+]{e^-} {}^{\bullet}OH \xrightarrow[H^+]{e^-} H_2O$$

**Figure 1.16 Reduction of molecular oxygen to water *via* ROS.**

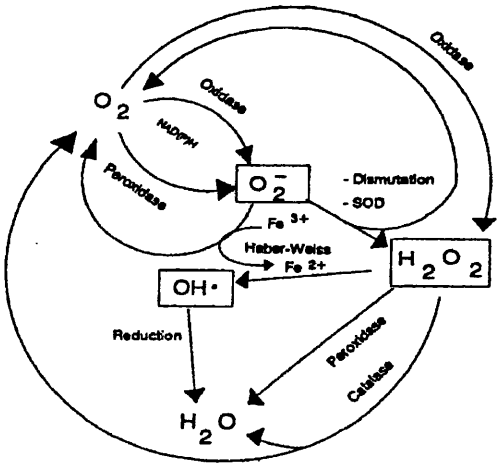


**Figure 1.17 Potential interconversion of ROS in biological systems (from Baker (1991)).**

Ground state $^{\bullet}O_2^-$ is a triple-state molecule and is relatively unreactive and non-toxic, but can become reactive and potentially dangerous to biological systems when its electron structure is altered (Baker & Orlandi 1995). In aqueous solution, the superoxide radical ($^{\bullet}O_2^-$), exists in equilibrium with its conjugate acid, the hydroperoxyl radical ($HO_2^{\bullet}$), which is a more hydrophobic molecule than superoxide and therefore more able to penetrate the lipid bilayers of membranes. In aqueous solutions, $^{\bullet}O_2^-$ disproportionates to $H_2O_2$ and $O_2$ (Wojtaszek 1997). This reaction occurs spontaneously or can be catalysed by superoxide dismutase (SOD), which is found in the cytosol, chloroplasts, and mitochondria (Scandalios 1993).

$H_2O_2$ is a relatively stable molecule resulting from the one electron reduction of superoxide or a two-electron reduction of molecular oxygen. It is able to pass through cell membranes (Apostol et al. 1989). Plant cells can remove $H_2O_2$, through the action of catalase and peroxidase (e.g. ascorbate and glutathione peroxidase). $H_2O_2$ can be disproportionated spontaneously, or by catalase, to form water and molecular oxygen. $H_2O_2$ can also be used as a substrate by various peroxidases in the formation of lignin, or be detoxified by ascorbate peroxidase *via* the Halliwell-Asada pathway. Additionally, $H_2O_2$ can be generated by peroxidases in reactions involving the formation of compound III ($Fe^{II}$-O-O-) (Wojtaszek 1997). Several enzyme activities can lead to the production of $H_2O_2$, one of which is xanthine oxidase (XO) (Slusarenko & Milosevic 1995). (Montalbini 1992) suggested xanthine oxidase to be responsible for the increase in the production of $^{\bullet}O_2^-$ and $H_2O_2$ after the infection of bean (*Phaseolus vulgaris*) with *Uromyces phaseoli*. Moreover, allopurinol, which is strong inhibitor of xanthine oxidase, also inhibited the

hypersensitive reaction (HR). The results from (Slusarenko & Milosevic 1995) on the interaction between *Phaseolus phaseolicola* and *P. syringae* were very similar to those of (Montalbini 1992), and furthermore they suggest that the production of ROS *via* XO is an important contributor to HR cell collapse and presented a possible model for the interaction **(Figure 1.18)**

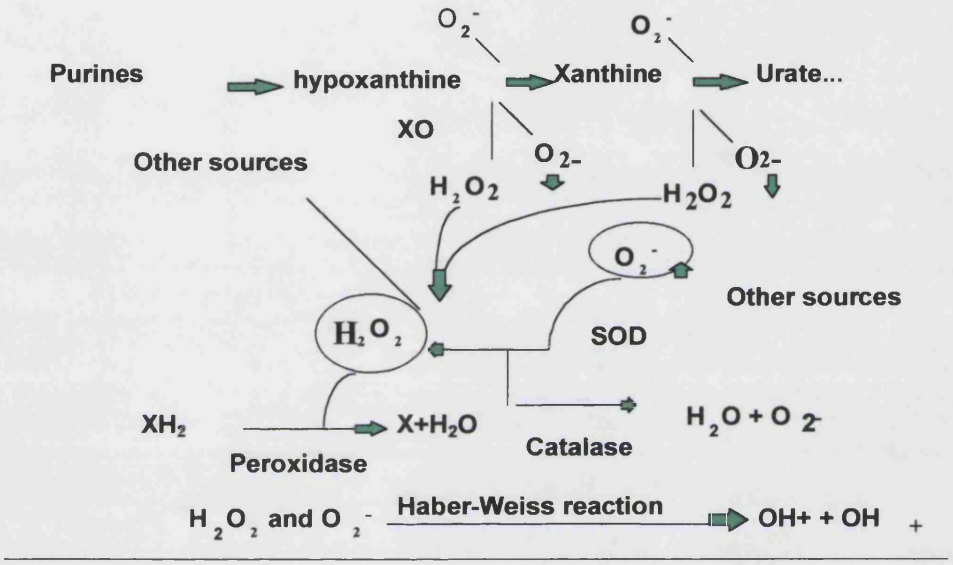


**Figure 1.18 Xanthine oxidase as a possible source of reactive oxygen species (after Slusarenko & Milosevic (1995)).**

$^{\bullet}OH$ it is the most reactive among ROS with a half-life in the range of microseconds. Due to its ability to initiate radical chain reactions, $^{\bullet}OH$ is considered to be the major factor responsible for the irreversible modifications of cellular macromolecules, the damage of organelles due to lipid peroxidation, enzyme inactivation and nucleic acid degradation (Lamb & Dixon 1997). It can be formed in different ways, including the oxidation of transition metals (e.g.

$Fe^{2+}$ or $Cu^{+}$) known as the Fenton reaction, or by the direct reaction of $H_2O_2$ and $^{\bullet}O2^{-}$ (Haber-Weiss reaction) (Wojtaszek 1997).

Several models of ROS synthesis during the plant oxidative burst have been suggested (Bolwell & Wojtaszek 1997; Doke 1995; Lamb & Dixon 1997; Low & Merida 1996). Comparative biochemical studies revealed two distinct mechanisms. Cultured cells of rose (*Rosa damascena*) treated with an elicitor derived from *Phytophthora* sp., produced hydrogen peroxide by a plasma membrane NADPH oxidase (superoxide synthase) system. Whereas, suspension culture cells of French bean (*Phaseolus vulgaris*), treated with an elicitor derived from cell wall of *Colletotrichum lindemuthianum*, produced hydrogen peroxide from cell wall peroxidases. In addition, cryptogein stimulation resulted in activation of an NADPH mechanism in rose suspension cells, whereas arginine stimulation activated the peroxidase system in bean suspension cells (Bolwell et al. 1998). However, the generation of ROS may be due to a combination of mechanisms. In tobacco leaves both mechanisms of ROS generation have been detected (Allan & Fluhr 1997). The principal models for the mechanism of reactive oxygen species production in plants are the NADPH-system (analogous to that of animal phagocytes), and the pH-dependent generation of $H_2O_2$ by cell wall peroxidases **(Figure 1.19)**.

Oxidative burst in plants was suggested to be mediated by an NADPH oxidase-system with strong analogies to the NADPH oxidase system of mammalian cells (Auh & Murphy 1995; Doke & Miura 1995; Lamb & Dixon 1997; Low & Merida 1996). This model states that an elicitor molecule is

recognised by a receptor located on the plasma membrane, and that the consequent cascade leads to the activation of NADPH oxidase for the generation of $^{\bullet}O_2^-$ and its dismutation to $H_2O_2$ (Legendre et al. 1993) The elements for this activation are several, including ion channels (Nürnberger et al. 1994), protein kinases, and protein phosphatases (Schwacke & Hager 1992), phopholipases A and C and GTP-binding proteins (Legendre et al. 1993). The existence of GTP-binding proteins and inositol triphosphate-mediated transduction was observed in soybean (*Glycine max* L.) cells in response to oligogalacturonides (Legendre et al. 1993). Evidence in favour of this model comes from work made on soybean, where the oxidative burst is inhibited by diphenylene iodonium (DPI), was blocked by protein kinase inhibitors, and was stimulated by protein phosphatase inhibitors. Additionally, an antibody to a component of the human oxidase (p22) reacts with a polypeptide of similar molecular weight in plant cell membranes (Lamb & Dixon 1997). Elicitation of tobacco cells by cryptogein leads to a decrease in NADPH concentration but not NADH, coincident with the synthesis of ROS. There is also a change in metabolites, indicative of the activation of the pentose pathway, which is the major source of NADPH. Inhibition of the pentose phosphatase pathway reduced the production of ROS by elicited cells as well as changes in the extracellular and intracellular pH, suggesting that supply of NADPH was essential for the oxidative burst (Pugin et al. 1997)

An alternative model of the generation of $H_2O_2$ by pH-dependent cell wall peroxidases has been proposed (Bolwell et al. 1995). This model states that the elicitor molecule is recognised by an appropriate receptor at the cell surface and that this event triggers ion channels. Movement of ions ($Ca^{2+}$, $K^+$, $H^+$, $Cl^-$)

results in a transient alkalinisation of the exocellular matrix (ECM) which leads to an activation of pH-dependent cell wall-bound peroxidases **(Figure 1.19)**. Generation of $H_2O_2$ by peroxidases is strongly pH-dependent with a maximum production between pH 7-9 depending on the peroxidase isoform (Bolwell & Wojtaszek 1997). French bean (*Phaseolus vulgaris* L.) suspension cells, elicited by the cell wall glucans of *C. lindemuthianum* produces an increase of the pH in the extracellular medium co-ordinately with the production of $H_2O_2$ (Bolwell et al. 1995). Evidence presented by (Bolwell et al. 1991) suggests that the mechanism that generates ROS in French bean suspension cells is validated by the inhibition of the oxidative burst by MES buffer or ionophores that prevent the alkalisation of the apoplast and by the inhibition of the burst by cyanide and azide (inhibitors of peroxidases). Moreover, transferring the cells to a buffered higher pH medium was sufficient to induce production of $H_2O_2$ (Wojtaszek 1997)

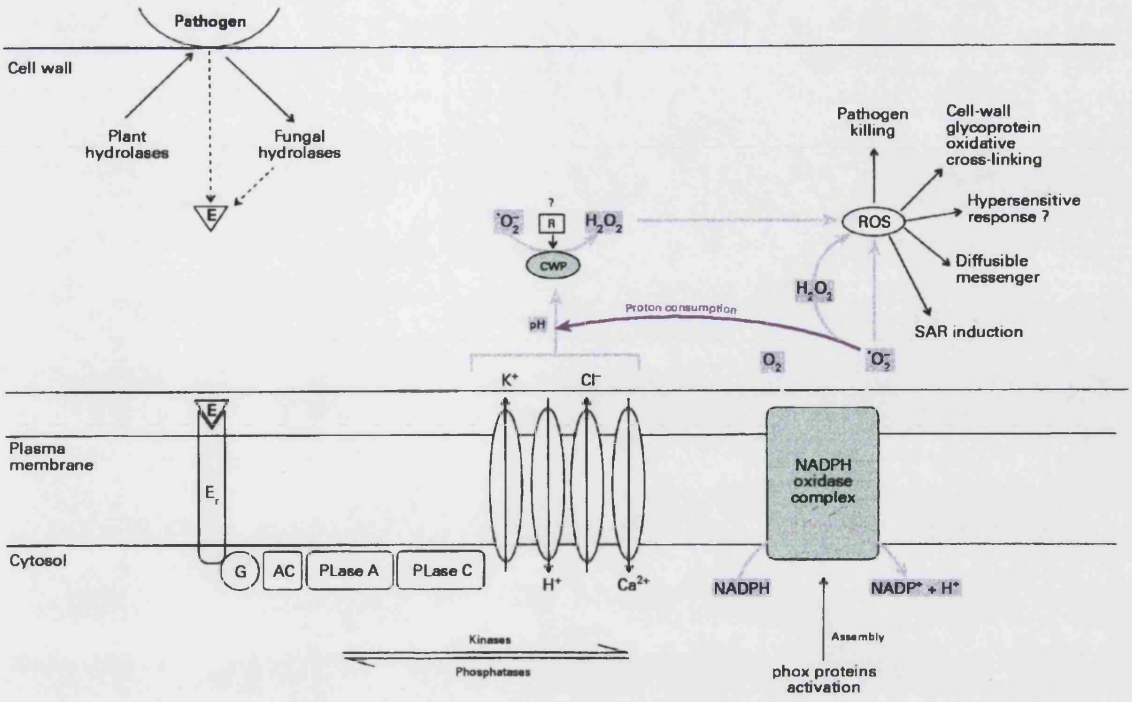


**Figure 1.19 Speculative model of components involved in ROS generation and effects of ROS (from Wojtaszek (1997)).**

**AC**: Adenylate cyclase; **CWP**: Cell-wall-bound peroxidase; **E**: Elicitor; **$E_r$**: Receptor; **G**: GTP-binding protein; **PLase A** and **C**: Phospholipase A and C; **R**: Reductant.

#### 2.1.4.3 Hypersensitive Response

One of the first responses of the plant at the site of attack is the hypersensitive response (HR). HR is observed after the incompatible interaction of plants with pathogenic viruses, bacteria or fungi as the development of lesions that restrict pathogen growth and spread. In interactions with obligate biotrophic pathogens, plant cell death would deprive the pathogen of further nutrients. The role of HR with hemibiotrophic and necrotrophic pathogens is less clear, however decompartmentalisation may lead to the release of anti-microbial compounds from preformed substances to the detriment of pathogens (Hammond-Kosack & Jones 1996). As a part of HR, plant cells at the site of

infection, suffer water-loss, electrolyte-loss, bleaching and rapid cell death and an array of defensive products are synthesised in adjacent cells (Graham & Graham 1999; Halverson & Stacey 1986). In most cases, activation of the HR leads to the death of cells at the infection site, which restrict the pathogen to small areas immediately surrounding the initially infected cells. However, several *R-Avr* gene-mediated resistances appear not to involve HR (Hammond-Kosack & Jones 1996). For instance, in parsley suspension cells infected with *P. infestans* a translocation of plant nucleus and cytoplasm was observed towards the site of infection and no HR was reported (Gross et al. 1993). The rapid cell death around the site of infection, and localised necrosis after pathogen recognition are genetically determined events, since mutants have been isolated that form HR-like necrotic lesions in the absence of pathogen infection, so called "paranoid plants" (Dietrich et al. 1994). The genes identified by these mutations (*acd*, for accelerated cell death, and *lsd*, for lesions stimulation disease) probably function downstream of the R genes in triggering HR (Dong 1995).

It has been proposed that the rapid cell death in plants resembles apoptosis (programmed cell death) in animals. On the other hand, the cytology of the HR in lettuce was more comparable to necrosis than apoptosis (Bestwick et al. 1995). The mechanisms underlying HR formation are not fully understood. However, it seems that plant cell death can arise due to either a switch in plant cell metabolism to biochemical changes that produce compounds or free radicals that are toxic to the pathogen and the plant cell. On the other hand, pathogen-derived products can trigger programmed cell death in plant cells (Hammond-Kosack & Jones 1996).

Associated with HR there exists a diverse group of genes that play direct or indirect roles in containing the pathogen. Products of these genes lead to the synthesis of a range of compounds that are involved in plant-resistance components, such as cell wall polymers (lignin, and suberin), as well as phenylpropanoids and phytoalexins. Additionally, some pathogenesis-related (PR) genes are induced rapidly in adjacent living cells by an avirulent pathogen during a HR. For example, ß-1,3-glucanases (PR-2) and chitinases (PR-3), and massive increases in the generation of reactive oxygen species (ROS) are also induced during the HR (Durner et al. 1997). Milosevic & Slusarenko (1996) used primary leaves of the Red Mexican bean plant inoculated with an avirulent isolate of *P. syringae* pv. *phaseolicola* to investigate the changes in enzymes related to the HR process. They concluded that acidic peroxidase, xanthine oxidase and glutathione reductase were up-regulated, and catalase down-regulated in tissues undergoing HR cell collapse. Atkinson et al. (1985) compared the response of tobacco suspension cells and tobacco plants exposed to incompatible bacteria (*P. syringae* pv. *pisi*) and found that the HR was similar in both systems. In both cases the appearance of characteristic symptoms of HR such as electrolyte efflux, respiratory stimulation, and the development of brown pigments occurred. These were followed by cell death.

## 2.1.5 Later Responses

### 2.1.5.1 Pathogenesis-Related Proteins

Pathogenesis-related proteins (PR) are a heterogeneous collection of proteins that are induced by several factors such as pathogen attack (bacteria, virus and fungi) or other stresses (wounding, hormones, ethylene, salicylic acid, elicitors, UV light) in both monocotyledons and dicotyledons plants (Collinge & Slusarenko 1987; Pegg & Young 1981) (Pegg & Young 1982; Popp et al. 1997; Stintzi et al. 1993; Ward et al. 1991). They are present in vacuoles, cell walls and intercellular spaces (Hason & Havir 1981). Acidic PR proteins generally accumulate in the cell-wall and basic PR-proteins in the vacuole (Walton 1997).

Initially, PR proteins were defined as a group of acidic-type proteins that accumulated after tobacco plants were infected by tobacco mosaic virus (TMV) (Vanloon & Vanstrien 1999). However, subsequently they have been reclassified as follows:

**PR-1**, whose biological activity is still unknown, but some members of which have shown to have antifungal activity and all are extracellular (Niederman 1995)

**PR-2** contains structurally distinct classes of β1,3-glucanases, as several isoforms are both extracellular and vacuolar. These proteins can hydrolyse fungal cell wall components that are mainly composed of glucan and chitin (Ryals et al. 1996a). These enzymes have been shown to liberate elicitor-active compounds (such as oligosaccharides) from fungal cell walls, which are able to trigger plant defence responses (Keen & Yoshikawa 1983; Stintzi et al. 1993). Additionally, it has been suggested that certain isoforms of PR-2 digest fungal cell walls specifically, and other isoforms digest plant cell walls (Hoj &

Fincher 1995; Okinaka et al. 1995). Recent reports indicate that over-expression of β1,3-glucanase in tobacco transgenic plants can enhance resistance to pathogenic fungi (Zhu et al. 1994). Although these proteins are important in defence processes in plants, they are also implicated in developmental processes (e.g. cell division), pollen germination, fertilisation, and seed germination (Beffa & Meins 1996).

**PR-3** consists of various kinds of chitinases, some vacuolar and others extracellular, and it seems their expression is regulated by complex tissue, developmental and environmental determinants (Lawton et al. 1992). They are considered as fungitoxic due to their capacity to degrade chitin (a β1,4- linked polymer of *N*-acetyl-β1,4-D-glucosamine) structural component of the fungal cell wall (Jung et al. 1993; Legrand et al. 1991; Pegg & Young 1982). Also, they can act as a lysozyme against bacterial walls (Boller et al. 1983). The chitinase function is co-ordinately regulated with β1,4-glucanase in tobacco cell culture tissues and it is inhibited by the plant hormones auxin and cytokinin (Shinshi et al. 1987).

**PR-4** it is the least studied class of PR proteins, they are of unknown activity and function

**PR-5** contains acidic-neutral and very basic members with extracellular and vacuolar localisation, respectively. All members show analogy to the sweet protein thaumatin (from *Thaumatococus daniellii*). They are probably antifungal based on evidence showing their *in vitro* capacity of inhibiting hyphal growth or spore germination of various fungi and by the formation of pores in the fungal membrane (Abad et al. 1996; Stintzi et al. 1993).

So far at least 33 different PR-proteins have been isolated and characterised in tobacco plants; 25 members of five major groups are also found in other plant species, although their function has not been determined for all of them (Stintzi et al. 1993). Additionally, there are other proteins whose induction has been related to pathogen attack, and which present antifungal activity. For example, thionins, α-amylases, and polygalacturonase-inhibiting proteins (PGIPs)

#### 2.1.5.2 Systemic Acquired Resistance

Systemic acquired resistance (SAR), is characterised by conferring to the plant a broad physiological immunity against a wide spectrum of microorganisms as a result of prior exposure to a necrogenic pathogen (Sticher et al. 1997). Resistance due to SAR is expressed locally at the site of primary inoculation but also systemically in tissues remote from the initial infection. Induction of resistance in parts remote from the site of primary inoculation is postulated to result from the translocation of a signal, which has not so far been conclusively identified. The signal is graft-transmissible and probably moves in the vascular system (Lucas 1998). However, the protection conferred by SAR is not passed on to seed progeny or transmitted to clonal tissues (Sticher et al. 1997).

The time needed for the establishment of SAR depends on both the plant and the type of inducing organism. There may even be a delay of up to several days between induction and full expression of SAR. But the protection conferred by SAR is long-lasting, often for weeks or even months, and this protection is non-specific, in the sense that it is effective against pathogens

unrelated to the inducing agent (Sticher et al. 1997). Use of *Arabidopsis thaliana* mutants and yeast two-hybrid experiments, has allowed some progress to be made towards identifying components of the SAR signal transduction pathway (Cao et al. 1994; Delaney 2000; Durner et al. 1997). A speculative model for the induction of SAR has been suggested (Schneider et al. 1996) **(Figure 1.20)**. Elicitors are released and perceived by a plant receptor, leading to the activation of a membrane bound NADPH-oxidase *via* a phosphorylation cascade. The reactive oxygen species produced by the NADPH system or membrane-bound peroxidase (not shown in the figure), cause an oxidative burst which may affect the pathogen directly, lead to cross-linking of cell wall proteins, or regulate the expression of host genes *via* a signal transduction pathway.

Both microorganisms and chemicals can induce SAR. For example, the addition of salicylic acid (SA), or its synthetic analogues [2,6-dichloroisonicotinic acid (INA), or benzo [1,2,3] -thiadiazole-7-carbothioic acid S-methyl ester (BTH), to plants induces SAR, and pathogenesis-related (PR) protein gene expression (Kessmann et al. 1994; Xie et al. 1998). Neither INA nor BTH cause an accumulation of salicylic acid, a key intermediate molecule in the SAR signal transduction pathway. When these compounds were applied to *NahG* transgenic plants (plants that enzymatically degrade SA they activated SAR by a pathway independent of the one involving SA in tobacco, *Arabidopsis*, and wheat, suggesting that INA and BTH act independently or downstream of SA in SAR signalling (Ryals et al. 1996b). However, BTH induces the same set of SAR genes (PR-genes) as those induced by SA (Friedrich et al. 1996; Lawton et al. 1995; Ward et al. 1991). However, this interpretation of the NahG transgenic experiments has been criticised as simplistic by Cameron (2000)

who pointed out that *NahG* plants have a phenylpropanoid pathway defect in addition to their inability to accumulate SA, which can affect the production of antimicrobial phenolics and lignin.

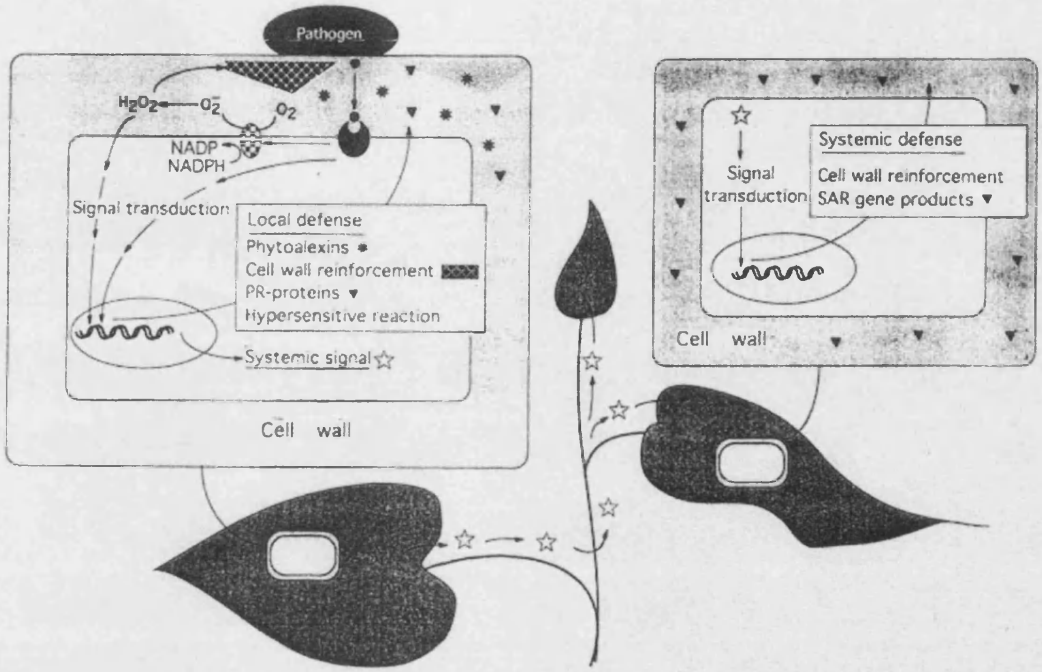


**Figure 1.20 Model for the induction of SAR (from Schneider et al. (1996)).**

Matthysse (1983) suggested that studies of acquired resistance *in vitro* with suspension cultured cells would be impossible because of the lack of intercellular and structural organisation inherent in such systems. Nevertheless, it has been reported that the pre-treatment of parsley suspension cells with SA, 2,6-dichloroisonicotinic acid (Thulke & Conrath 1998), or BTH (Katz et al. 1998) sensitise parsley cells to elicitors in a process known as "conditioning". Conditioned tobacco suspension cells respond with many of the principal features observed in the expression of SAR genes in the whole plant (Xie et al.

1998). In addition, it was shown that, not only coumarin accumulation was influenced by SAR activators, but also the elicitor-induced incorporation of phenolic compounds into the cell wall, and the generation of reactive oxygen species occurred (Kauss & Jeblick 1995). The addition of SA enhanced the elicitor-induced expression of genes coding for phenylalanine ammonia-lyase (PAL), 4-coumarate: CoA ligase (4CL), PR-10 and hydroxyproline-rich glycoprotein (HRGP). On the other hand, anionic peroxidase (POD), and mannitol dehydrogenase (MTD) encoding genes were directly activated by salicylic acid in the absence of elicitor. As a consequence of these findings, a dual role for salicylic acid (Thulke & Conrath 1998), and BTH (Katz et al. 1998) in the activation of defence genes in parsley cells was proposed. Thus, SA may activate the pathway that leads to the induction of SA-responsive genes in parsley, for example POX and MTD, while it potentiates other genes such as those for PAL, 4CL, PR-10, and HRGP genes.

#### 2.1.5.3 Phenylpropanoid Metabolism and Phenylalanine Ammonia-Lyase

In plants a major flow of carbon from primary to secondary metabolism occurs *via* general phenylpropanoid metabolism (Lamb & Dixon 1994). Phenylpropanoid compounds play important role in the control of many processes such as plant development, growth, xylogenesis, and flowering (Jones 1984b). Additionally, many aspects of defence that are activated by pathogens, elicitors or wounding derive from the general phenylpropanoid pathway, of which phenylalanine ammonia-lyase (EC 4.3.1.5) (PAL) is the key entry point diverting the flux of phenylalanine from protein to phenolic biosynthesis **(Table 1.7)**.

PAL, catalyses the elimination of ammonia and pro-3S hydrogen from L-phenylalanine to form *trans*-cinnamate; this product can lead to different branch pathways, of which some end products are related to defence responses in plants such as lignin, phytoalexins and UV protectants **(Figure 1.20)**. Several compounds had been reported to be synthesised in response to pathogen attack, including pterocarpans, isoflavans, prenylated isoflavonoids, stilbenes, psoralens, coumarins, flavonols and aurones that derive from general phenylpropanoid metabolism (Dixon & Lamb 1990). Additionally, use of PAL inhibitors reduces defence responses. For instance, S-carvone inhibits PAL activity and suberisation during wound healing of potato tubers (Oosterhaven et al. 1995).

The PAL enzyme is a tetramer with a molecular weight (MW) with range of 240,000 to 330,000 Daltons dependent upon the species, but can be dissociated into subunits of MW~ 55,000 to 85,000 (Hason & Havir 1981). PAL subcellular localisation is mainly cytoplasmic, although it may also associated with some membranous organelles (Bolwell & Rodgers 1991; Jones 1984a). PAL is encoded by a multigene family with 2 to 6 members in most plant systems studied (Cramer et al. 1989; Hahlbrock & Scheel 1989). However, over 40 PAL genes has been reported in potato, and only one in loblolly pine (Joos & Hahlbrock 1992; Whetten & Sederoff 1992). Southern blot analysis indicates there are at least three genes in cassava, cDNA and genomic clones of which have been isolated (Beeching et al. 1998). PAL isoenzymes exist as products of a small (3-4) gene family in bean (*Phaseolus vulgaris* L.) (Bolwell et al. 1985), parsley (*Petroselium crispum* L.) (Lois et al. 1989), rice (*Oryza sativa* L.) (Zhu Q et al. 1995) and *Arabidopsis thaliana* (Wanner et al. 1995). Despite

distinct isoenzymes of PAL being present in cell suspension cultures of fungal-treated alfalfa (*Medicago sativa* L.), no specific distinctive function for any of these isoenzymes has yet been identified and thus, the reasons for their formation remains unclear (Davin LB & Lewis NG 1992).

PAL is sensitive to the physiological state of the plant or suspension cells, and changes in activity can occur during growth stages (Jones 1984a). Furthermore, increases in PAL occur in response to a wide variety of stimuli including dilution of suspension cultures (Hahlbrock 1976; Messner & Meinrad 1993), the action of light (white, red/far red or blue/UV) (Hahlbrock & Ragg 1975; Schröder et al. 1976; Zimmermann & Hahlbrock 1975), to several elicitors (Bach & Seitz 1997; Bolwell & Rodgers 1991; De Lorenzo et al. 1987; Dixon & Lamb 1979; Edwards et al. 1985; Fritzemeier et al. 1987; Kervinen et al. 1998; McGhie et al. 1997), plant growth regulators (Dixon & Fuller 1976), mechanical stress or exposure to ethylene (Babic et al. 1993), wounding (Diallinas & Kanellis 1994; Joos & Hahlbrock 1992), cold-stress and other stresses (Ni et al. 1996; Solecka 1997), infection (Corchete et al. 1993; Cui et al. 1996) and methyl jasmonate and salicylic acid (Sharan et al. 1998; Szabo et al. 1999; Smith-Becker et al. 1998).

RNA blot hybridisation and nuclear run-off experiments showed that elicitor, wounding, infection and irradiation stimulate the accumulation of PAL mRNA and that this coincided with increases in enzyme synthesis and activity in several systems (Edwards et al. 1985). For instance, *de novo* synthesis of PAL mRNA was induced after 2.5 hours in elicitor-treated bean suspension cells (Edwards et al. 1985). However, the transcription of PAL genes can occur within 10 minutes after elicitation with a fungal cell wall preparation (Hedrick et

al. 1988). The ability to increase or reduce the expression of specific genes of phenylpropanoid metabolism leads to major understanding of their importance in defence. For example antisense suppression of 4-coumarate: coenzyme A ligase activity in *Arabidopsis* results in plants with up to 50% decreases in lignin content (Lee et al. 1997).

| Stress factor | Plant part | Phenylpropanoids accumulated | References |
|---|---|---|---|
| Pathogen (*C. fimbriata* f.sp. *platani* | Plantain suspension culture | Xanthoamol (dihydrofuranocoumarin) | Alami et al. (1998) |
| Wounding | Tomato leaves | E-feruloyltyramine (hydroxycinnamate-tyramine conjugates | Pearce et al. (1998) |
| Wounding | Carrot | Phenolics (p-hydrobenzoic acid) | Babic et al. (1993) |
| UV-irradiation | Carrot suspension culture | Anthocyanins | Ozeki & Takeda (1994) |
| Low temperature | Maize seeds | anthocyanins | Christie et al. (1994) |
| Nutrient deficiency (nitrogen-lack) | Alfalfa roots | flavonoids | Coronado et al. (1995) |

**Table 1.7 Selected examples of stress-induced phenolic compounds.**

### 2.1.5.4 Phytoalexins

Many biochemical changes occur in plants in response to stress, attack by pathogens or exposure to elicitors. These changes include the production of new compounds induced by such challenges. Since (Müller & Borger 1940) presented the phytoalexin hypothesis based on their work with the

*Phythopthora infestans* / potato plant interaction, several definitions had been suggested. A general definition of phytoalexins is that they are low molecular weight antimicrobial compounds that are both synthesised *de novo* (from distant precursors) and accumulate in plants in response to stress, elicitors or microbial infection (Dixon et al. 1983; Hammerschmidt 1999; Kuc 1995; Whitehead & Threlfall 1992) **(Table 1.8)**. In metabolic terms, phytoalexins are secondary metabolites that are distinguished from chemically related compounds by their antimicrobial property and the attributes of synthesis *de novo* in a plant tissue following infection or stress (Whitehead & Threlfall 1992).

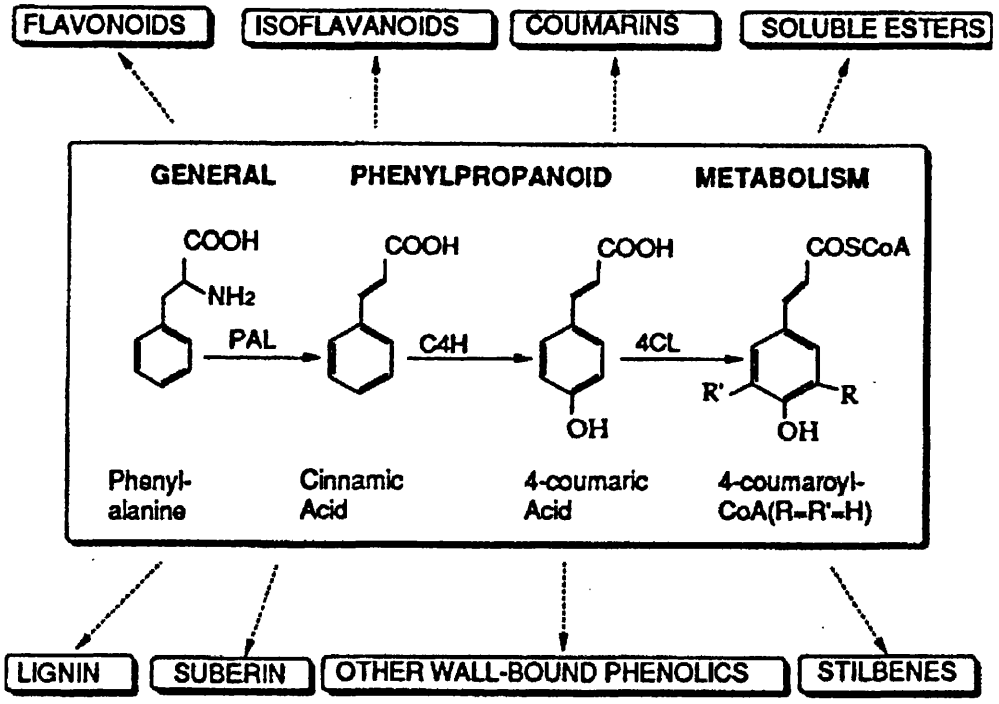


**Figure 1.21 General phenylpropanoid metabolism (from Douglas et al. (1992)).**

**PAL**, Phenylalanine ammonia-lyase; **C4H**, Cinnamate 4-hydroxylase; **4CL**, 4-Coumarate:CoA Ligase.

So far, more than 350 phytoalexins have been chemically characterised from approximately 30 plant families. Although most phytoalexins have been isolated from dicotyledons, there are some examples from monocotyledons including rice, corn, sorghum, wheat, barley, onion and lilies (Kuc 1995). However, there exist families that show limited induction of phytoalexins (e.g. *Cucurbitaceae* and *Rosaceae* families), perhaps due to their possession of alternative barriers to pathogens (Harborne 1999). Phytoalexins are compounds with chemically diverse structures, but they can be classified into four main chemical classes, phenolics, acetylenics, terpenoids and nitrogen-containing compounds (Harborne 1999). Biosynthetically, phytoalexins can be derived from one or several biosynthetic pathways such as the shikimic acid, acetate-mevalonate, acetate-malonate (Dixon et al. 1983). Notwithstanding their diversity in chemical structure, plant families produce phytoalexins that fall into the same chemical classes, such as terpenoids of the *Solanaceae* family. In fact, secondary metabolites have been used as a basis for plant classification (Hammerschmidt 1999).

Usually, phytoalexins accumulate differentially within the tissues of a plant (Mansfield 1983). Furthermore, relative proportions of the different phytoalexins accumulating in a given plant species may depend upon the nature of the invading pathogen (Bailey 1987). Based on comparative biochemical analysis of diverse kinds of phytoalexins in plants, (Harborne 1999) pointed out that the type of phytoalexin produced is likely to be related to existing pre-infectional agents present in a specific family. He concluded that the only feature that phytoalexins have in common is a significant degree of lipophilicity.

As phytoalexins have been found in most plants, a number of general biological features can be summarised. There is a close correlation between phytoalexin accumulation and necrogenic (hypersensitive) resistance to fungal, bacterial and viral infections. The accumulation of phytoalexins during the hypersensitive response is modulated by a number of factors including ontogenic changes in the host, temperature, light intensity and photoperiod, phytohormone concentrations, carbon and nitrogen status, ultraviolet irradiation (Dixon et al. 1983; Dixon & Fuller 1976).

| Plant | Elicitor | Phytoalexin formed | Reference |
|---|---|---|---|
| Carrot | Yeast extract | 6-methoxymellein | Guo & Ohta (1993) |
| Apple | Yeast extract | Aucuparin | Borejsza-Wysocki et al. (1999) |
| French bean | *Colletotrichum lindemuthianum* | Phaseollin | Dixon & Lamb (1979) |
| Soybean cells | Glucan from cell walls of *Phytophthora megasperma* f.sp. *glycinea* | Glyceollin | Ebel et al. (1984) |
| Chili pepper | cellulase | Capsidiol | Chávez-Moctezuma & Lozoya-Gloria (1996) |
| London plane tree (*Platanus x acerifolia*) | Glycoprotein from *Ceratocystis fimbriata* | Umbelliferone | Alami et al. (1998) |
| Parsley | 13-amino acid oligopeptide fragment from *Phytophthora sojae* | Furanocoumarins | Jabs et al. (1997) |

**Table 1.8 Selected examples of phytoalexins formed in plant suspension cells challenged with elicitors.**

The question arises as to whether phytoalexins are involved in defence or are just a consequence of resistance conferred by other means. Evidence of the role of phytoalexins in defence comes from the localisation and timing of phytoalexin accumulation close to the infected tissues, phytoalexin production linked to a rapid restriction of pathogen development, and a positive relationship between pathogen virulence and tolerance to phytoalexins (Bennett & Wallsgrove 1994). A strong correlation between rapid phytoalexin biosynthesis and disease resistance was shown when roots of a resistant cultivar of soybean were infected with *P. megasperma* f. sp. *glycinea*. The phytoalexin glyceollin I was induced to toxic ($EC_{90}$) levels within eight hours in the resistant cultivar rather than 14 hours later as occurred in a susceptible cultivar, and the development of the fungus was stopped in the resistant variety soon after penetration of the epidermal layer (Hahn et al. 1985). In most cases phytoalexins have been localised to the tissue at the site of bacterial or fungal infection. For instance, the resistance of cocoa (*Theobroma cacao* L.) to *Verticillium dahliae* Kleb was associated with the localised production of sulphur phytoalexin in the vessels and surrounding parenchyma cells; a localisation that strongly indicated that this phytoalexin could play an important role in defence against this xylem-invading pathogen (Resende et al. 1996). Another approach to evaluating the role of phytoalexins is with molecular tools. For instance, transforming plants with sense constructs of phytoalexin biosynthetic genes, or with antisense constructs (as has been accomplished with other defence-associated genes) to affect the capacity of the plant to produce phytoalexins, thereby enabling to see if these changes can be correlated with altered susceptibility to infection (Rogers et al. 1996). This approach has only recently

been envisaged since several enzymes that appear to be phytoalexin pathway-specific have been identified. Genes encoding some of these have been cloned and these findings could play an important part in the understanding of phytoalexin function in plants. For example, in the Solanaceae and Malvaceae, sesquiterpenoid phytoalexins are produced. Recent identification and cloning of the gene for the first enzyme specifically required for sesquiterpene production, (a sesquiterpene cyclase) that converts farnesyl diphosphate (C15) into cyclic hydrocarbon, has been reported for several plants (Kuc 1995). Further evidence comes from tobacco *via* the engineering of the phytoalexin resveratol by means of the constitutive expression of the terminal biosynthetic enzyme, stilbene synthase (Hain et al. 1993). These tobacco transgenic plants displayed enhanced resistance to the necrotrophic fungus *B. cinerea*. In addition, there exist good correlations between the amount of phytoalexin produced and the inhibition of pathogen growth such as for phaseollin accumulation in HR cells of bean responding to *C. lindemuthianum* (Dixon & Lamb 1979). However, the majority of the studies only give a correlation and do not replicate field conditions where diseases and stresses are far more complex (Hammerschmidt 1999).

While different plants produce different phytoalexins the capacity of pathogens to detoxify or show tolerance to phytoalexins is highly specific. For example, the potato dry rot pathogen *Gibberella publicaris* is able to detoxify the sesquiterpenoid phytoalexin (rishitin) of potato (Gardner et al. 1994). The pea pathogen *Nectria haematococca* detoxifies pisatin (phytoalexin) through a pisatin demethylase enzyme that produces the less toxic compound (+)-6a-hydroxymaackiain. (Lamb et al. 1992). The role of phytoalexins has been

presented only in terms of disease resistance. However, there is evidence that isoflavonoids, many of which are phytoalexins, stimulate the infection of plant roots by beneficial microbes and that mutualistic bacteria elicit the exudation of some classical phytoalexins. This implies a more flexible definition of these compounds than as exclusively antimicrobial (Hammerschmidt 1999). For example, the root exudate of alfalfa (*Medicago sativa* L.) inoculated with symbiotic *Rhizobium meliloti* bacteria produces a conjugated form of the medicarpin precursor formononetin 7-O- (6"-O-malonylglycoside) which induces *nod* gene (nodulation genes) transcription in *R. meliloti* (Dakora et al. 1993).

Despite their importance in aspects of resistance, plant-pathogen interactions cannot be explained exclusively by the presence of phytoalexins. These compounds are only one component in the complex mechanism of defence (Hammerschmidt 1999).

## 3 DEFENCE AND STRESS RESPONSES IN CASSAVA AND OTHER MEMBERS OF THE *EUPHORBIACEAE* FAMILY

Hitherto, stress and defence-related responses have been little studied in the *Euphorbiaceae* family. Most of research which can be related to stress and defence-related responses in cassava has been concentrated on two of its major problems, the high content of cyanogenic glycosides, and the abiotic stress, post-harvest physiological deterioration (PPD), of the roots. Toxicity due to cyanogenesis can be a problem to consumers but the risk is easily eliminated by processing (Cock 1985). Cyanogenic plants are characterised by the liberation of toxic cyanide, HCN, by the action of β-glucosidases and hydroxynitrile lyases on cyanogenic glycosides. The enzymes and substrates

are kept separate in the plant cell and only come into contact with each other, and release HCN, when the plant is damaged (Vetter 2000). In cassava, there are two cyanogenic glycosides, linamarin and lotaustralin, which are produced from valine and isoleucine in the presence of NADPH in a process, involving P-450 enzymes (Koch et al. 1992).

Cyanogenesis is considered to have a protective role against herbivores, including grazing animals. In cassava roots the presence of cyanogenic glycosides had been reported to improve resistance to the cassava root borer (*Cyrtomenus bergi*) (Bellotti & Arias 1993). By feeding adults and nymphs, with a cyanogenic clone, the arthropod showed larger nymphal development, reduced adult longevity, reduced egg production and increased mortality. By contrast cyanogenic glycosides seemed not to have role in resistance against mealybug (*Phenacoccus manihoti*) in cassava leaves (Calatayud et al. 1996). Clear increases in the levels of rutin (a glycosyl flavonoid) but no modification in cyanide content occurred in leaves of cassava infected with mealybug. Conversely, HCN could be deleterious to the plant due to its capacity to inhibit tanning reactions (Mederacke et al. 1995). Furthermore, HCN that is liberated during the infection of leaves of the rubber tree (*Hevea brasiliensis*) with South American leaf blight (*Microcyclus ulei*) inhibits phytoalexin production (scopoletin) in neighbouring cells (Giesemann et al. 1986). They suggested that strongly cyanogenic *Hevea* species are much more sensitive to infecting fungi than weakly cyanogenic varieties. This cyanide tolerance by some fungi has been attributed to cyanide-resistant respiration. The majority of the pathogens, which can succeed in the infection of cyanogenic plants, have cyanide hydratase. This enzyme detoxifies HCN by converting it to formamide.

Apparently this particular enzyme appears to be specific to fungi, as it has not so far been found in bacteria (Osbourn 1996).

After harvest cassava roots deteriorate rapidly in a two stage process. During the first stage, which can occur within 24 hours of harvesting, the roots undergo physiological and biochemical changes which are characterised by the discoloration of vascular tissues, a process known as post-harvest physiological deterioration (PPD). Subsequently, microbial invasion of the root tissues occurs (Beeching et al. 1998). During PPD the activity of phenylalanine ammonia-lyase (PAL) increased in roots during the development of primary deterioration. The activity reached a peak 72 hours after harvesting but with enormous variation between cultivars of as much as ± 36 hours and coincided with an increase in phenolic compounds (Tanaka et al. 1983; Uritani et al. 1984).

Peroxidases and polyphenol oxidase also increase during root discoloration and are accompanied by increases in stress metabolites including flavonoids, catechins and terpenoids (Beeching et al. 1998; Rickard 1985). In particular, the coumarin scopoletin (6-methoxy-7-hydroxycoumarin), scopolin (6-methoxy-7-hydroxycoumaroyl-7-ß-D-glucoside), esculin (6,7-dihydroxycoumaroyl-6-ß-D-glucoside) and two conjugates containing scopoletin and esculin increase in abundance (Tanaka et al. 1983). Scopoletin was also reported as a compound formed in cassava roots after infection with *Pythium* sp. and *Botryodiploda theobromae* (Taniguchi & Data 1984). Scopoletin was also identified and associated with resistance mechanisms in the interaction between *Hevea brasiliensis* infected leaves with *Microcyclus ulei*, and *Colletotrichum gloesporioides* (García D et al. 1995; Giesemann et al. 1986). Twenty-two diterpenic compounds were isolated from wounded or fungal

infected cassava root tissues, although their biological activities were not determined. These compounds can be classified into four families, *ent*-beyerane, *ent*-pimarane, *ent*-atisane and *ent*-kaurane (Sakai & Nakagawa 1988).

*Ricinus communis* L. seedlings respond to challenges from a variety of fungi by producing an antifungal diterpene hydrocarbon, casbene (Lee & West 1981). The key enzyme casbene synthase which catalysed the synthesis of casbene from geranylgeranyl diphosphate has been cloned from *Ricinus* (Lois & West 1990). *Euphorbia calyptrata* suspension cells produce lactone diterpenoids such as helioscopinolides (Minghetti et al. 1996). Many members of *Euphorbiaceae* family produce abundant latex and that from *Hevea brasiliensis* contains chitinases and lysozymes, which are capable of degrading the chitin component of fungal cell walls and the peptidoglycan component of bacterial cell walls (Martin 1991). Cassava latex contains lysozyme, protease and glucanase activities and PAL transcripts (R.M. Cooper personal communication). Hevamine, a basic enzyme with lysozyme/chitinase activity from *H. brasiliensis* latex, may have a role in plugging the latex vessels and cessation of latex flow (Jekel et al. 1991). Several chitin-binding proteins were isolated from the "bottom fraction" of *H. brasiliensis* latex; one of these was hevein, a small cysteine-rich protein that showed strong antifungal activity against several fungi *in vitro* (Van Parijs et al. 1991). During bark regeneration of *H. brasiliensis* after tapping, tannin content in the rays and axial parenchyma of the outer region occurred and some deposition of callose in the perforations of the sieve elements adjacent to the wound was observed (Thomas et al. 1995). Callose deposition, increase in cinnamyl alcohol dehydrogenase (CAD,

EC 1.1.1.195), and lignin synthesis ocurred in *H. brasiliensis* after roots were challenged with elicitors obtained from *Rigidoporus lignosus* (Nicole et al. 1991). Also, lignification and suberisation were associated with callose deposition and secretion of phenolic compounds by tyloses in cassava cultivars infected with *Xam* (Kpémoua et al. 1996). Mbaye (1989) reported that 4-methylresorcinol, catechol, quercetin, and ferulic and ρ-coumaric acids extracted from infected cassava inhibited *Xam* or *Xac* growth.

## 4 ELICITORS OF PLANT DEFENCES

Elicitors are molecules that may originate from the pathogen or the host plant and which induce or trigger host defences. Many elicitors have been used to investigate plant-microorganism interactions **(Table 1.9)**. A basic classification of elicitors divides them into abiotic and biotic elicitors (Dixon 1986; Ebel & Mithöfer 1998). Molecules with elicitor activity have been identified with a wide range of chemical structures, including oligosaccharides (e.g. β-glucans), glycoproteins (e.g. from *P. megasperma*), lipids (e.g. arachidonic acid and eicosapentaenoic acid), lipopolysaccharides (e.g. protein-lipopolysaccharide elicitor from *V. dahliae*), proteins (e.g. elicitins), peptides (e.g. monilicolin A; Pep-13, flg22, flg15, systemin) and enzymes (e.g. xylanase) (Dixon 1986; Ebel & Cosio 1994; Gomez-Gomez et al. 1999; Halverson & Stacey 1986; Parker et al. 1991; Smith 1996; Smit & Dubery 1997; Stennis et al. 1998). Also, potent elicitors have been isolated from non-pathogens, such as the yeast *Saccharomyces cerevisiae*. For instance, yeast is an effective elicitor of phytoalexin synthesis in soybean (Baier et al. 1999; Guo et al. 1998; Hahn & Albersheim 1978).

Race specific elicitors, which induce a response only in host cultivars on which that race of the pathogen is avirulent, have been predicted to represent direct or indirect products of avirulence genes (Hammond-Kosack & Jones 1997; Keen 1986). For instance, a partially purified galactose/mannose-rich glycoprotein from the alpha race of *C. lindemuthianum* induced phytoalexins in a bean cultivar resistant to the alpha race, but not in a compatible cultivar (Tepper & Anderson 1986). Also a race-specific elicitor was isolated from intercellular fluids of compatible race-cultivar interactions of *C. fulvum* / tomato. This elicitor specifically induced chlorosis and necrosis in resistant but not in susceptible plants (De Witt & Spikman 1982). In addition, injection of elicitor preparations containing the *Avr9* gene product of race 4 of *C. fulvum* into tomato which carries the *Cf9* gene, induced leaf necrosis and reactive oxygen species (ROS) (Lu & Higgins 1998). However, the vast majority of elicitors are not race specific (Dixon 1986). The use of axenic cultures as a source material, and harsh treatments such as acid hydrolysis and heat solubilization in elicitor isolation, may account for the observed lack of race-cultivar specificity in the majority of preparations (Lamb et al. 1989).

### 4.1 ABIOTIC ELICITORS

Abiotic elicitors are molecules, which are not derived from natural sources such as the tissues of the pathogen or host. They include salts of heavy metals, e.g. $Cu^{2+}$ and $Hg^{2+}$, detergents such as sodium dodecyl sulphate (SDS) and Triton X-35, fungicides, basic molecules such as polylysine and histone, solvents such as chloroform, and molecules that can intercalate DNA such as 5-bromo-deoxyuridine and 9-aminoacridine (Darvill & Albersheim 1984;

Dixon et al. 1983; Smith 1996). Other factors that can cause abiotic stress include temperature-shock, wounding, and exposure to UV light (Smith 1996).

### 4.2 BIOTIC ELICITORS

Biotic elicitors are divided between those that originate from the invading organism, so-called "exogenous" or "microbial", and "endogenous" which are of plant origin and are generated by the plant-pathogen interactions (Smith 1996). Based on the nature of elicitor-release different kinds of biotic elicitors have been suggested. Thus, exogenous elicitors can be structural components of the pathogen or metabolites that are secreted into the environment by pathogen (Ebel & Mithöfer 1998). For example, an elicitor may be released directly by the microorganism (West 1981), or released from the plant by microbial enzymes (Lee & West 1981), or plant enzymes may liberate cell wall components from the micro-organism (Côté et al. 1995; Ham et al. 1995) **(Figure 1.22)**, and finally endogenous elicitors released by the plant which are activated in response to various stimuli such as SA and JA, which act as secondary messengers in plants (Draper 1997; Lyon et al. 1995).

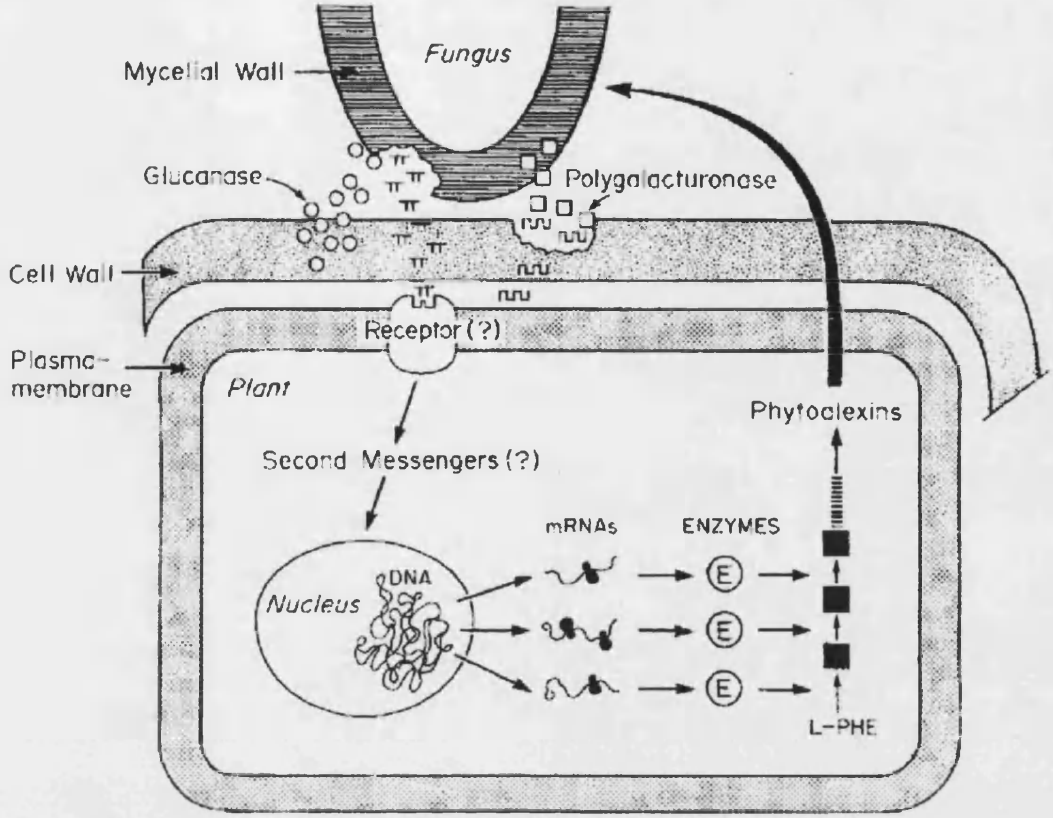


**Figure 1.22 Origin of elicitors from microbiological source (from Hahn et al. (1994)).**

Oligosaccharide elicitors were the earliest to be characterised. Four major classes of oligosaccharides elicitor have been identified: oligoglucan, oligochitin, oligochitosan of fungal origin and oligogalacturonide of plant origin (Cote & Hahn 1994). The best characterised glucan elicitor was isolated from race 3 of mycelial cell walls of *Phytophthora megasperma* f. sp. *glycinea*; it was determined that a specific branched (1,3-1,6)-hepta-β-glucoside fragment is the minimal elicitor-active structure (Sharp et al. 1984) **(Figure 1.23)**. This elicitor is effective at concentrations as low as $10^{-9}$-$10^{-10}$ M at inducing glyceollin production (phytoalexin) in soybean cotyledons (Darvill & Albersheim 1984).

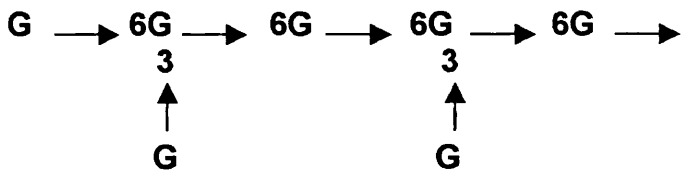


**Figure 1.23 Structure of the smallest elicitor-active, β-3,6-heptaglucoside fragment obtained from mycelial wall of *P. megasperma* (after Darvill & Albersheim (1984)).**

**G**: Glucose

The fact that subtle changes in structure of this elicitor reduced activity, led to the experiments towards the identification of specific elicitor-receptors in plants. Elicitor-binding proteins with a high degree of specificity have been localised within soybean plasma membranes (Hahn & Cheong 1991). The successful cloning of a gene coding for a receptor in the plasma membrane of soybean root cells of the β-glucan elicitor from *P. megasperma* has been reported (Umemoto et al. 1997), although it is not yet known if this binding protein is the elicitor receptor (Klüsener & Weiler 1999). Glucan elicitors were not released from fungal cell walls in the absence of contact with the host. For example, host β-1,3-endoglucanase isoenzymes hydrolyse the β-1,3-glucans from the pathogen hyphal cell wall (Keen & Yoshikawa 1983). Conversely, it had been reported that *P. megasperma* f. sp. *glycinea* secretes an inhibitor of soybean isoforms of β-1,3-endoglucanase (Ham et al. 1997).

French bean suspension cultures challenged with a crude elicitor from cell walls of *C. lindemuthianum* accumulated the isoflavonoid phytoalexin

(phaseollin) coincident with an increase in *de novo* synthesis of PAL (Dixon & Lamb 1979). In various elicitor preparations, activity has been associated with a fraction rich in carbohydrate, containing galactosyl, glycosyl and mannosyl residues as common constituents (Smith 1996). In the glycoprotein fraction from *C. lindemuthianum* the carbohydrate moieties were found to be responsible for activity. Whereas with parsley suspension cells, the protein portion of a 42-kDA glycoprotein or even an oligopeptide fragment of 13 amino acids (Pep-13) from *P. sojae* were responsible for activity (Nürnberger et al. 1994; Sacks et al. 1995). A receptor / ligand complex in the parsley system has been visualised on the protoplast surface by incubation with the elicitor from *P. sojae* (Pep-13 ) and silver enhanced immunogold labelling antibodies as viewed by epipolarization microscopy (Diekmann et al. 1994).

Chitin is a major component of fungal cell walls. It constitutes a linear polymer of (1,4)-linked N-acetyl-β-glucosamine, and the smallest size detected with elicitor-active capacity is the tetramer (Barber & Ride 1994). Chitin elicitors triggers an alkalinisation of the extracellular medium, oxidative burst and phytoalexin synthesis in tobacco suspension cells (Baier et al. 1999). Chitosan, a partially deacetylated derivative of chitin, is able to activate plant defence responses in plants such as pea, parley, tomato, and rose that are not responsive to chitin fragments (Ebel & Mithöfer 1998). Suspension cultured barley cells responded to chitosan elicitor with an increase in PAL activity and of wall-bound phenolics 2-4 hours after elicitation (Schiffer et al. 1997). The minimum oligomer size required for elicitor-active chitosan was determined in a study with chemically-synthesised oligomers, in which the octamer induced pisatin accumulation in pea tissues, but longer oligomers were not tested in

these studies (Hadwiger et al. 1994). More specific detail of oligomer size was obtained studies on rice where the highly elicitor-active (1nM) *N*-acetylchitooctaose was obtained, however oligomers smaller than the trimer showed no activity (Kuchitsu et al. 1997). Although, a specific receptor for chitosan and chitin elicitors has not been found, the requirement for a definite oligomer size for biological activity of these elicitors suggests that a receptor is a part of their mechanism of action (Smith 1996).

Other exogenous elicitors are arachidonic acid (AA) and eicosapentaenoic acid (EPA), which are present in the cell walls of *Phytophthora* spp. These polyunsaturated fatty acids, which are race non-specific elicitors, induced browning and sesquiterpenoid phytoalexin accumulation (capsidiol and rishitin) in green pepper suspension cells (Hoshino et al. 1994). Optimum elicitor activity was achieved with C20 fatty acids, however high concentrations ($\geq$ 0.3mM) of AA are needed to elicit phytoalexin accumulation in potato tubers. These molecules alone are probably not physiologically important elicitors, although they are thought to act synergistically with other *Phytophthora* glucan elicitors (Halverson & Stacey 1986).

Bacteria can also produce exogenous elicitors. For instance, *E. amylovora* contains the *hrpN* gene encoding harpin. Harpin is a cell-envelope associated protein of 44 kDa that elicits a hypersensitive response in the host plant accompanied by $K^+$ and $H^+$ ion exchange (Wei et al. 1992). Peptides such as flg22 and flg15 corresponding to eubacterial flagellin, the protein that builds up the filament of eubacterial flagella, act as potent elicitors in tomato leaf discs,

in which they caused the oxidative burst and callose deposition (Felix et al. 1999).

Endogenous elicitors that originate from the plant following interaction with pathogens, mostly appear to be apoplastic and to modulate the extent of responses in the surrounding tissue (Ebel & Cosio 1994). This modulation can be achieved independently of the presence of exogenous elicitors or in a synergistic manner (Darvill & Albersheim 1984). Oligalacturonides derived from plant cell wall pectin with a degree of polymerisation (DP) higher than nine are able to elicit phytoalexin synthesis in soybean cotyledons (Cervone et al. 1989), and in castor bean seedlings (Jin & West 1984). These elicitors derived from pectic polysaccharides which comprise 1,4-linked α-D-galactosyluronic acid residues, and constitute key structural components of the primary cell walls of higher plants. Jasmonic acid causes protease inhibitors and ribosome-inactivating proteins to accumulate (Kauss et al. 1992). Salicylic acid appears to play an important role in defence against pathogen attacks, particularly in signalling after elicitation by other molecules. Salicylic acid accumulates at lesions formed as a result of the hypersensitive response (Draper 1997). Endogenous glutathione may also be involved in signal transduction by fungal elicitors during the initiation of phytoalexin formation as a defence response in legumes (Edwards et al. 1991).

| Source | Elicitor | Biological effect | Reference |
|---|---|---|---|
| | **Exogenous** | | |
| *Colletotrichum lindemuthianum* | Glycoprotein (Mr 28,000) | Phaseollin in bean (phytoalexin) | Coleman et al. (1992) |
| **Erwinia amylovora** | Protein (Mr 44,000) (harpin) | Necrosis in tobacco | Wei et al. (1992) |
| *Phytophthora megasperma* f.sp. *glycinea* | Hepta-(1→3,1→6)-β-glucoside | Glyceollin in soybean (phytoalexin) | Sharp et al. (1984) |
| | **Endogenous** | | |
| **Glycine max** | Oligo-α-(1→4)-galacturonate | Glyceollin (phytoalexin) | Nothnagel et al. (1983) |
| **Ricinus communis** | Oligo-(1→4)-αgalacturonate | Lignin synthesis (reinforcement cell wall) | Bruce & West (1989) |

**Table 1.9 Some examples of elicitors of plant defence responses (after Ebel & Cosio (1994))**

## 5 *IN VITRO* APPROACHES TO STUDYING DEFENCE-RELATED GENES AND PRODUCTS

Haberlandt (1902) was the first to carry out pioneering experiments on maintaining plant cells in a viable state away from the parent body by submerging them in simple nutrient solutions. In the mid 1950's attention was drawn to the possibility of using plant cells on an industrial scale for the synthesis of natural products and in the 1970's there was a renewed upsurge of interest, from the new biotechnology companies. Plant cell culture has frequently been considered as an alternative to agricultural production for a wide range of compounds such as pharmaceuticals, flavours, fragrances, and

colours. In addition, plant suspension cultures can be used in biotransformations for the production of specific enzymes (Scragg 1990).

The advantages of suspension cells are several. The cells are morphologically more homogeneous than undifferentiated leaves or roots, and thus likely to show a more uniform and rapid biochemical response to elicitor action than the heterogeneous cell populations of intact plants (Masuta et al. 1991). Cells have greatly reduced cell-to-cell interactions, which helps to elucidate biochemical pathways (Barz et al. 1990). Usually addition or removal of cells or solution samples can be performed in a non-destructive and facile manner. Frequently, lack of chlorophyll and polyphenols, which are present in the whole plant, facilitates the extraction of proteins, DNA and RNA. Also, many parameters can be controlled precisely under aseptic conditions such as temperature, media components and light (Barz et al. 1990; Dixon 1980; Ozeki & Takeda 1994). Furthermore, plant cell cultures are independent of seasonal, geographical, and political problems. In addition to their potential exploitation in commerce, plant tissue culture has various advantages over whole plants for more fundamental research.

They became a model system of choice for dissecting many plant responses using current molecular and biochemical methods (Scheel 1998). In fact, much of understanding plant-pathogen interactions comes from such studies. Several aspects of plant defence signal transduction have been elucidated using plant suspension cultures challenged with elicitors. For instance, the ion fluxes across the plasma membrane (Scheel 1998), the production of reactive oxygen species (ROS) (Apostol et al. 1989; Auh & Murphy 1995; Bolwell et al. 1998), the induction of cell death (Gustine et al.

1995; Koch et al. 1998), the production of hydrolytic enzymes (e.g. chitinase and glucanase) capable of attacking surface polymers of the pathogens (Messiaen & Van 1993; Popp et al. 1997; Umemoto et al. 1997), the synthesis of proteins capable of inhibiting degradative enzymes produced by the pathogens (PGIPs) (Bergmann et al. 1994; De Lorenzo et al. 2000), the modification of plant cell walls by the deposition of callose, the accumulation of HRGPs and lignin (Boudart et al. 1995; Grosskopf et al. 1991; Kauss 1994; Kawalleck et al. 1995; DeBuck et al. 2000; Sommer-Knudsen et al. 1998), and the detection of *de novo* synthesised antimicrobial compounds (phytoalexins) (Borejsza-Wysocki et al. 1999; Davis et al. 1998; Whitehead & Threlfall 1992). Significant correlations between *in vitro* and *in vivo* responses have been reported. For example, a correlation between *in vivo* resistance to pathogens and *in vitro* increases of phenylpropanoid metabolism and defence responses following elicitor-treatment of plant suspension cells, have been found in different plant-elicitor combinations (Bolwell et al. 1985; Desikan et al. 1996; Dixon et al. 1981; Edwards et al. 1985; Jabs et al. 1997; Scheel et al. 1994; Thulke & Conrath 1998).

Notwithstanding all the above mentioned advantages of suspension cells, the biological properties and responses of these systems have been criticised as not representing organised plant material and in some instances disease resistance or susceptibility of the whole plant have not been reflected at the level of suspension cells. For instance, in soybean callus cultures where the extent of glyceollin (phytoalexin) production by callus culture of different host cultivars was quantitatively different from that of the intact plant (Keen & Horsch 1972). By contrast, capsidiol (phytoalexin) were found in tobacco callus

as well as in tobacco plants (Bailey 1975). It is more than 20 years since (Dixon 1980) stated that further work was required to define more fully the similarities and differences between cell cultures and their tissues of origin, however there is still remaining a gap in the information on several systems between tissue culture and the whole plants. However, for example, while it has been claimed that it is not possible to study SAR in plant cell suspension culture (Matthysse 1983), others disagree (Thulke & Conrath 1998). While cell suspension cultures and elicitor systems may not provide universal answers, they remain a powerful tool of choice for the dissection of many key aspects of plant defence responses. Therefore, they were used in this study as a key approach to begin to elucidate some putative components of cassava defence responses. Ultimately, it is essential to confirm important aspects *in planta* and some attempts to do this will also be described.

# CHAPTER 2

# MATERIALS AND METHODS

## 1 CASSAVA TISSUE CULTURE

### 1.1 PLANT MATERIAL

Stakes of cassava cultivar MCol 22 were provided by the Centro Internacional de Agricultura Tropical (CIAT) in Cali, Colombia, and were propagated in a glasshouse under the following conditions. The stakes were cut into 15-20 cm lengths and dipped in a commercial rooting powder containing IBA (Seradex, May and Baker Ltd.). The stem cuttings were planted in a mixture of peat and perlite (3:1 v/v) in 12 cm diameter plastic pots. The stems were maintained initially at high humidity (80%) and 28-30°C. When the stem had developed roots and shoots, stems were transferred to 20 cm diam pots that contained John Innes No.2 compost and a slow release fertiliser (Vitafeed 101). Plants were grown under glasshouse conditions with a photoperiod of 12 h with natural lighting supplemented by mercury lamps from October to March, with a temperature range of 28-30°C and 80% humidity.

## 1.2 TISSUE CULTURE TECHNIQUES

All the chemical reagents in this materials and methods chapter were from Sigma unless otherwise stated.

### 1.2.1 Culture Media

The basal medium Murashige and Skoog (MS) was used for the induction and maintenance of plantlets, callus and cell suspension cultures of cassava (Murashige & Skoog 1962). For plantlets, the medium did not contain 2,4-dichlorophenoxyacetic acid (2,4-D) and for cell suspension cultures it did not contain agar. The medium contained MS salts (Flow laboratories, U.K.), sucrose (BDH Chemicals LTD., Poole, Dorset, U.K) 20 g/l, 2,4-D at 1, 1.5, 2, 2.5, 3, 3.5, 4 mg/l, and agar 0.7% (w/v) (Oxoid Cat. No. 3) and the pH was adjusted to 5.8 with 1 M NaOH. Sterilisation was by autoclaving at 1.5 bar/120°C for 15 min. Subsequent procedures were carried out in the sterile environment provided by a laminar flow cabinet (Microflow Servicecare Ltd., Andover, Hampshire, U. K.). Approximately 20 ml of the medium was dispensed into 9 cm diam. plastic sterile Petri dishes (Bibby Sterilin Ltd., Stone Staffs, U. K.), and 40 ml of medium into sterile jars. When the medium solidified the Petri dishes or the sterile jars were stored in the dark at room temperature until required.

1.2.2 Establishment of Plantlets

All the steps below were carried out in a laminar flow cabinet. Young green stem nodes of approx. 2 cm length from MCol 22 were submerged in sodium hypochloride 10% (v/v) for 10 min. Then explants were washed three times with sterile distilled water (SDW) for 1 min. each time. Finally, explants were placed on sterile filter paper Whatman No.1, (Whatman Ltd, U. K.), and the ends of the internodes were cut off with a sterile blade and discarded, after which the explants were cultured on solid medium in sterile jars in order to obtain the plantlets. Explants cultured on solid medium, and callus cultures, were incubated at 25 ± 2°C on shelves illuminated by fluorescent lamps (30 μm/S/PAR) in a culture room with a 16 hour photo-period.

1.2.3 Establishment of Callus Cultures

From the plant material in the sterile jars, petioles of 1 to 1.5 cm lengths were taken and placed into new sterile Petri dishes. These segments were placed into Petri dishes containing solid medium and the boxes sealed with parafilm M (American-Canadian Company, U.S.A.). After three weeks, by which time callus had grown at the ends of the petioles, the callus was cut off and placed into Petri dishes containing fresh medium for at least three weeks more. After that period of time the most soft, friable callus was subcultured onto fresh medium as required at monthly intervals.

1.2.4 Initiation and Maintenance of Suspension Culture from Callus

The callus obtained was passed through a sterile metallic mesh, with pore size of about 0.05 mm. Five g were transferred into sterile flasks containing 50 ml of liquid MS medium. The medium was supplemented with 2% sucrose, and 2 mg/l 2,4-D. Flasks were incubated at 110 rpm and 25 ± 2°C in the dark for 3 or 4 weeks in a shaking incubator (Model G25, New Brunswick Scientific Co. Inc, U.S.A.). After three weeks, the cell suspension was established and regularly subcultured. The subculture was initiated every 7 days by transferring 10 ml of the cell suspension into sterile flasks containing 45 ml of fresh medium.

Two days before each subculture, the medium and the suspension cells were tested for contamination by fungi and bacteria. Under the aseptic conditions of a laminar flow hood, five drops of cassava suspension cells were added to a Petri dish containing potato dextrose agar (PDA)[1] or nutrient agar (NA)[2]. The plates were sealed with Parafilm and incubated at 26°C for two days. Growth indicated contamination.

[1] Potato Dextrose Agar
39 g of PDA powder (Bury, England) was added to 1 litre of SDW. The preparation was autoclaved at 1.5 bar/120 °C for 15 min.

[2] Nutrient Agar
25 g of Nutrient Broth No.2 and 12 g of tissue culture agar Mc29, was added to 1 litre of SDW. The preparation was autoclaved at 1.5 bar/120 °C for 15 min.

### 1.2.5 Assessment of Suspension Culture Growth

The growth of cell suspension cultures was monitored by the measurement of the packed cell volume (PCV). PCV was monitored by transferring all the content of each flask to 50 ml sterile plastic tubes, and centrifuging in a bench centrifuge for 5 min at 2,500 x g, and determining the pellet volume as a function of the volume of the culture.

### 1.2.6 Viability Assessment of Cassava Suspension Cells

#### 1.2.6.1 FDA Method

Cassava suspension culture cells was mixed in a haemocytometer with 0.5% (w/v) of fluorescein diacetate (FDA) and then examined after 10 min under the fluorescence microscope (Widholm 1972). The exciting light wavelength was in the blue region at $490_{nm}$. The living cells gave a green fluorescence and dead cells did not fluoresce.

### 1.2.7 Measurement of pH in Cassava Suspension Cells

The pH was measured with an Orion Research digital ionalyzer/501 pH meter.

# 2 ELICITORS

## 2.1 EXOGENOUS ELICITORS

### 2.1.1 Yeast Elicitor

Initially the elicitor was kindly provided by Dr. R. Dixon (Samuel Roberts Noble Foundation, Oklahoma, U.S.A). However, subsequently yeast glucan cell wall elicitor was prepared by the method described by (Schumacher et al. 1987). Two kg of fresh baker's yeast was suspended in a 4 l sodium citrate buffer (20 mM; pH 7.5) and autoclaved at 1.5 bar at 120°C for 60 min. This preparation was centrifuged for 20 min at 10,000 x g at 4°C. One volume of ethanol, 95% (v/v), was added to the supernatant. After stirring slowly overnight at 4°C the precipitate was removed by centrifugation 20 min at 10,000 x g at 4°C. One volume of ethanol 95% (v/v) was added to the supernatant drop by drop. After stirring overnight at 4°C the supernatant was removed by centrifugation for 20 min at 10,000 x g at 4°C and the precipitate was freeze-dried at –20°C for two days. Finally, the pellet was stored at –20°C until use.

In order to determine the glucose equivalents of the elicitor a standard curve with D-glucose was made according to (Dubois et al. 1956) **(Figure 2.1)** using D-glucose 0.5 mg/ml; aqueous phenol 10% (w/v), and concentrated sulphuric acid (95.5%), which were mixed in glass tubes. These tubes were left to stand at room temperature for 10 min and then incubated at 30°C for 20 min, before determining absorbance at $490_{nm}$ in a microplate reader (DYNATECH MR 5000). Three independent replicates were used.

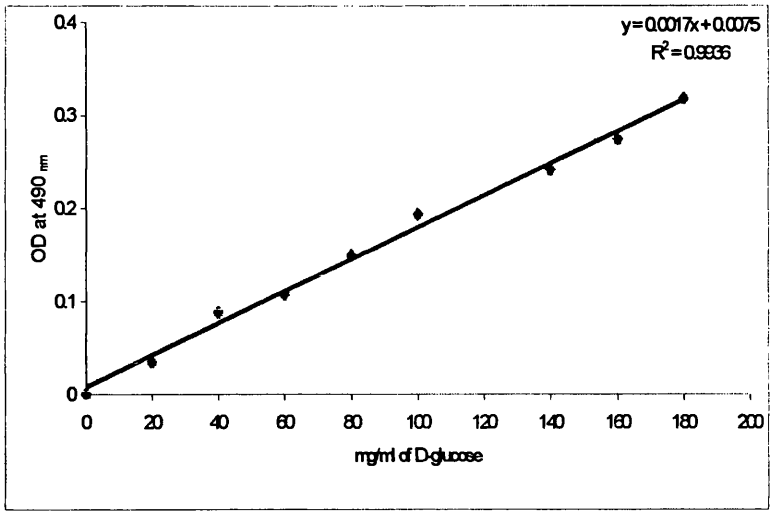


**Figure 2.1 Standard calibration curve for glucose determination by phenol-sulphuric acid.**

Glucose content determination of yeast elicitor by the phenol-sulphuric acid method was performed. One gram powder of yeast elicitor was dissolved in 10 ml of distilled water and filtered (Millipore MA 0.45 μM pore size). The yeast elicitor solution was assayed using the same protocol as for the standard curve described above. For each concentration three independent replicates were taken.

2.1.2 *Colletotrichum lindemuthianum* Elicitor

Dr. R. Dixon (Samuel Roberts Noble Foundation, Oklahoma, U.S.A.) and Prof. P. G. Bolwell (Royal Holloway College, University of London, England) kindly provided this elicitor. Cell wall glucan preparations from *C.*

*lindemuthianum*, had been obtained by the method described by (Dixon & Lamb 1979). This elicitor was dissolved in SDW before use.

### 2.1.3 Oligogalacturonic acid Elicitor

Dr. F. Cervone (Dipartimento di Biologia Vegetale, Università di Roma "La Sapienza", Italy) and Dr. J. Dow (Purdue University U.S.A.) kindly provided this elicitor. This elicitor was obtained by the method of (Nothnagel et al. 1983). This elicitor was dissolved in SDW before use.

### 2.1.4 Xanthan Gum Elicitor

Commercial Xanthan gum from *X. campestris* at 0.15% w/v in SDW was sterilised by ultraviolet light for two days before use as an elicitor with cassava suspension cultured cells.

### 2.1.5 Arachidonic Acid Elicitor

Arachidonic acid was dissolved in SDW before use.

### 2.1.6 Lipopolysaccharide Elicitors

Prof. K Rudolph (Göttenheim University, Germany) kindly provided these elicitors. These elicitors, from *Pseudomonas* and *Xanthomonas* (isolate St03) were obtained by the method of (Keen 1984). These elicitors were dissolved in SDW before use.

## 2.2 ENDOGENOUS ELICITORS

(±)-Jasmonic acid (JA); salicylic acid (SA); glutathione (GSH). Before use all these elicitors were dissolved in SDW, except jasmonic acid, which was dissolved in dimethyl sulfoxide (DMSO).

## 2.3 TREATMENT OF CASSAVA SUSPENSION CELLS WITH ELICITORS

Cassava suspension cells were treated with each elicitor after 5 days of subculture. Depending on the concentration of the elicitor used, the volume was adjusted, but never exceeded 10% of the total final volume in the flask. Control cells were treated with SDW. However, in the case of cells treated with JA, DMSO was added to the control cells as a solvent control.

### 2.3.1 Collection and Storage of the Cassava Elicited Cells

After elicitation the cells were harvested at time intervals, washed twice with 100 ml of SDW on a porous-glass funnel with filter paper (Whatman No.1)

and finally, frozen in liquid nitrogen and stored at -80°C until required. Control cells were similarly collected.

# 3 ENZYME ASSAYS

## 3.1 PROTEIN DETERMINATION OF CASSAVA CELLS AND MEDIA

Protein quantification for all the enzymes assayed was by the Bradford method and a standard curve was performed according to (Stoscheck 1990). A standard curve was made with bovine serum albumin (BSA) **(Figure 2.2)**. One mg of BSA was dissolved in 1 ml of SDW, then this solution was mixed with 1.5 M NaOH and reagent mix[3] in a microcentrifuge tube for each reaction. Before reading the absorbance at $595_{nm}$, in a microplate reader (DYNATECH MR 5000) the microcentrifuge tubes were placed at room temperature for 5 min. For each concentration three independent replicates were used.

[3]Reagent mix

0.07% Coomasie brilliant blue G in 100 ml of orthophosphoric acid, and 50 ml of absolute ethanol.

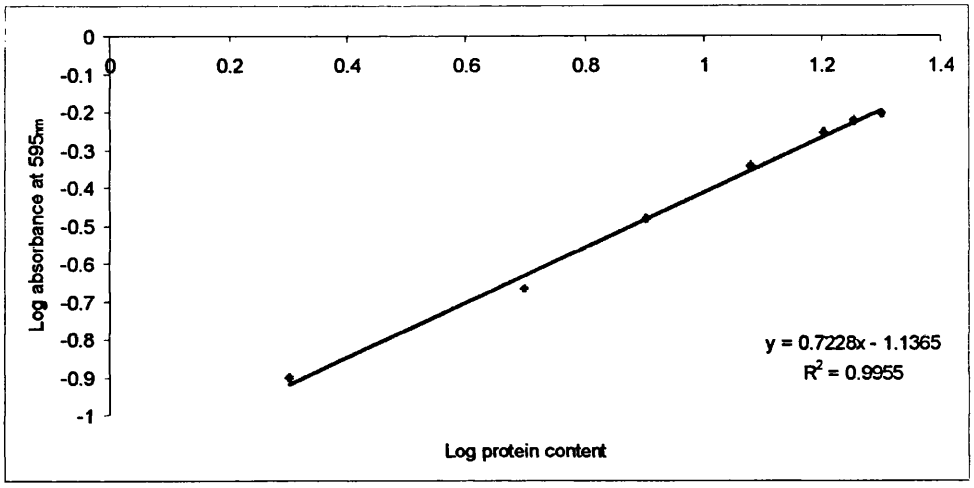


**Figure 2.2 Standard calibration curve for determination of protein concentration by the Bradford method.**

3.2 ENZYME EXTRACTION AND ASSAY FOR PHENYLALANINE AMMONIA LYASE

Crude enzyme extracts and enzyme activity assays were performed according to (Hahlbrock & Ragg 1975). Two grams of frozen cells were homogenised with 4 ml of 0.1 M sodium borate buffer pH 8.8 which contained 1.4 mmol/l of β-mercaptoethanol, with an ultra-turrax homogenizer (TP 18-10. IKA WERK, Staufen, Germany) for 30 min; the tubes were keep on ice during this time. Debris was then removed by centrifugation at 4°C for 15 min at 20,000 x g. The extracts were stored at –70°C until required for enzyme activity assays.

To perform the enzyme activity assay, 200 μl of the extract obtained as above was made up to 0.5 ml with 0.1 M sodium borate buffer (pH 8.8) and then added to an equal volume of the same buffer containing 0.02 M L-phenylalanine

or D-phenylalanine. The mixture was incubated at 30°C, and the absorbance at $290_{nm}$ was measured at 0, 15, 30 and 60 min in a CECIL 6600 Multimode computing UV spectrophotometer. Three independent experiments were done for each concentration and time course point.

### 3.3 ENZYME EXTRACTION AND ASSAY FOR XANTHINE OXIDOREDUCTASE

Crude enzyme extraction and the fluorimetric assay were done according to (Beckman et al. 1989). Cassava cell suspensions were transferred to sterile tubes and centrifuged at 1,000 x g at room temperature for 5 min. The supernatant was then discarded and the pellets were suspended in 1.2 ml of 0.05 M phosphate buffer which containing 0.1 mM EDTA, 0.1 μg ml/l each of pepstatin A, leupeptin, antipain and aprotinin. The mixture was transferred to a 1.5 ml sterile microcentrifuge tube and sonicated in a MSE 150 Watt Ultrasonic Disintegrator Mk2 for 20 secs on a power setting < 6.0. Finally, after sonification the samples were ultra-centrifuged in a Beckman LB50 ultracentrifuge, for 25 min at 4 °C at 35,000 x g. The extract was used immediately for determining the enzyme activity.

A fluorimetric assay for measurement of the enzyme activity was done in quartz cuvettes at dual wavelengths of 345 and $390_{nm}$. The base line was obtained using 480 μl of the previous buffer and 500 μl of crude extracts, after 5 min 10 μl of the reducing substrate, pterin, and 10 μl of the oxidising substrate methylene blue were added. After 10 min the reaction was stopped by the addition of 50 μl of allopurinol; at this point the calibration standard curve was made by the addition of successive 2 μl aliquots of isoxanthopterin and readings

taken after each addition. Three independent experiments were performed and three replicates were taken for each particular point.

### 3.4 ENZYME EXTRACTION AND ASSAY FOR PEROXIDASE

0.5 g of suspension cells were ground for 15 min in a pestle and mortar with 5 ml of 50 mM phosphate buffer, pH 6.0, 0.15% polyvinylpyrrolidone (PVP) insoluble, 1 mM EDTA, 1 mM dithiothreitol (DTT) (Acros), and 0.5 mM α-toluenesulfonyl fluoride 99% (PMSF) (Acros). The mortar was kept on ice during this time. Debris was then removed by centrifugation at 4°C for 45 min at 15,000 x g. The extract was used for enzyme activity assays. A standard curve was made with horseradish peroxidase (HRP) **(Figure 2.3)**.

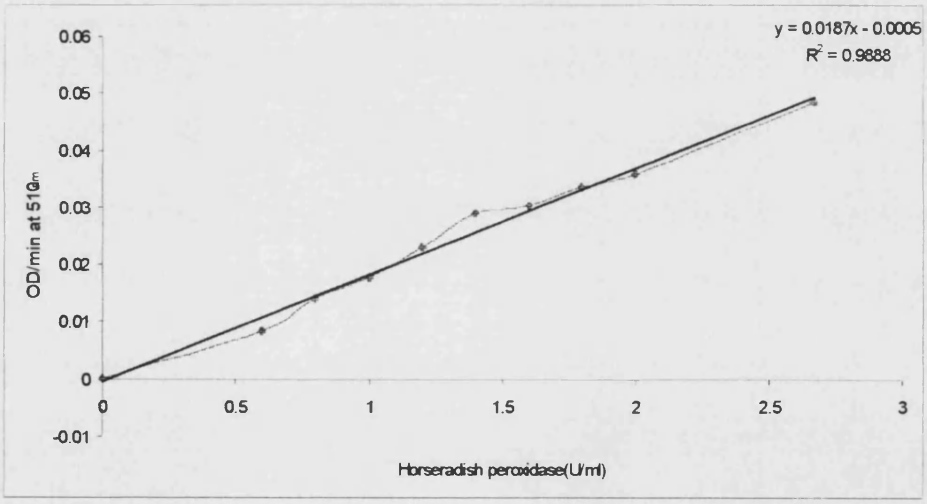


**Figure 2.3 Horseradish peroxidase standard calibration curve.**

Peroxidase activity was assayed by the method of (Van Gestelen et al. 1997). The 3.0 ml reaction volume contained 50 mM phosphate buffer (pH 6.0), 100 mM (3,5-dichloro-2-hydroxybenzensulfonic acid) (DHBS), 10 mM (4-

aminoantipyrine) (AA), and an aliquot of enzyme extract. After incubation at 25°C for 5 min, the reaction was started by the addition of 100 mM $H_2O_2$, and the reaction followed by the change in absorbance at $510_{nm}$ in a CECIL 6600 multimode computing UV spectrophotometer. Three independent experiments were done and in each case three replicates were taken.

# 4 ELECTROFOCUSING METHODS

## 4.1 PEROXIDASE ISOFORMS

### 4.1.1 Extraction Method for Symplasmic Peroxidases

The extraction method was identical to that used for enzyme activity, except 3 g of cells were used instead of 0.5 g. Solution volumes were adjusted accordingly. After centrifugation the supernatant was passed through a PD 10 (Sephadex G25 M) column (Amersham) and the solution was concentrated at 4°C for 2 h with polyethylene glycol 35,000 (PEG) in dialysis tubing.

### 4.1.2 Extraction Method for Extracellular Peroxidases

Cells after elicitation were left in a laminar flow cabinet for 15 min to sediment. 15 ml of medium were collected into Falcon tubes (50 ml capacity) by filtration through three layers of miracloth membrane, and then frozen in liquid nitrogen before stored at –70°C. Frozen samples were thawed on ice in the cold room overnight, then centrifuged at 10,000 x g. The supernatant placed in a dialysis tube with molecular weight cut-off 12-14000 Daltons (Medicell International Size 2 Inf. Dia. 18'/32) and dialysed for 2 days against 10

mM phosphate buffer pH 6.0. The buffer solution was replaced every 24 h. The samples were concentrated at 4°C for 6 h with polyethylene glycol 35,000 (PEG) in dialysis tubing.

### 4.1.3 Electrofocusing Conditions for Peroxidase Isoforms

Peroxidase isoforms were detected by the method of (Manchenko 1994). Electrofocussing was performed on a Multiphor II Electrofocusing LKB BROMMA 2127 system (Pharmacia Biotechnology). The Multiphor II electrophoresis unit was cooled down for 20 min at 16°C. Afterwards, 1 ml of paraffin was put in the equipment, and then the plastic template 125 x 260 mm was located in place. The electrophoresis tanks were filled with 100 ml of freshly prepared 1 M NaOH to absorb $CO_2$, which may affect the pH of the electrode solutions (cathode and anode), during the run.

An Ampholine PAG polyacrylamine gel, with pH ranges 3.5-9.5, was located over the plastic template. Electrode strips (cathode and anode) were carefully positioned over the edges of the gel after they had been soaked in electrode solutions. The cathode electrode strip was soaked in 3 ml of 1 M NaOH and the anode electrode strip was soaked in 3 ml of $H_3PO_4$. 5 μg of protein for each sample was pipetted onto the sample application paper. The broad pI calibration kit (pH3-10) was used. A standard calibration curve was made according to the manufacturer's instructions **(Figure 2.4)**. A vial containing the marker was reconstituted with 100 μl of SDW. 10 μl of the reconstituted solution was pipetted onto the sample application paper. The gel was run at 1,500 V and 50 mA with the power (W) setting of 30 for 45 min. The

sample application papers were then removed and the gel run for a further 35 min. After running, the pI markers was cut from the gel and stained with Coomasie blue according to manufacturer's instructions. The remainder of the gel was then used for detection of protein isoforms by direct staining of the gel until the red-brown bands appeared[4]. Peroxidase isoforms with activity toward scopoletin was obtained staining the IEF peroxidase isoforms gel with 0.1 mM scopoletin dissolved in 0.1 M phosphate buffer pH 6.0 (Gutierrez et al. 1995).

[4] Fixing solution for pI marker
29 g of trifluoroacetic acid (TFA) mixed with 8.5 g sulphosalicylic acid and dissolved in 250 ml of SDW. Fix pI marker for 60 min at room temperature.
Staining solution for pI marker
0.5% Brilliant Blue R, (w/v) in destaining solution. Stain pI marker for 10 min at room temperature.
Destaining solution for pI marker
500 ml of ethanol mixed with 160 ml acetic acid. Make up to 2 l with SDW. Destain pI marker until the background is clear.
Preserving solution for pI marker
25 ml of glycerol mixed up to 250 ml of destaining solution. Preserve pI marker for 60 min at room temperature.
Staining solution for peroxidase isoforms
50 mM phosphate buffer (pH 6.0), 100 mM (3,5-dichloro-2-hydroxybenzensulfonic acid) (DHBS), 10 mM (4-aminoantipyrine) (AA), 100 mM $H_2O_2$. The gel was soaked in this staining solution at 4 °C until red-brown bands appeared. Immediately pictures were taken and the distances of the bands were measured.

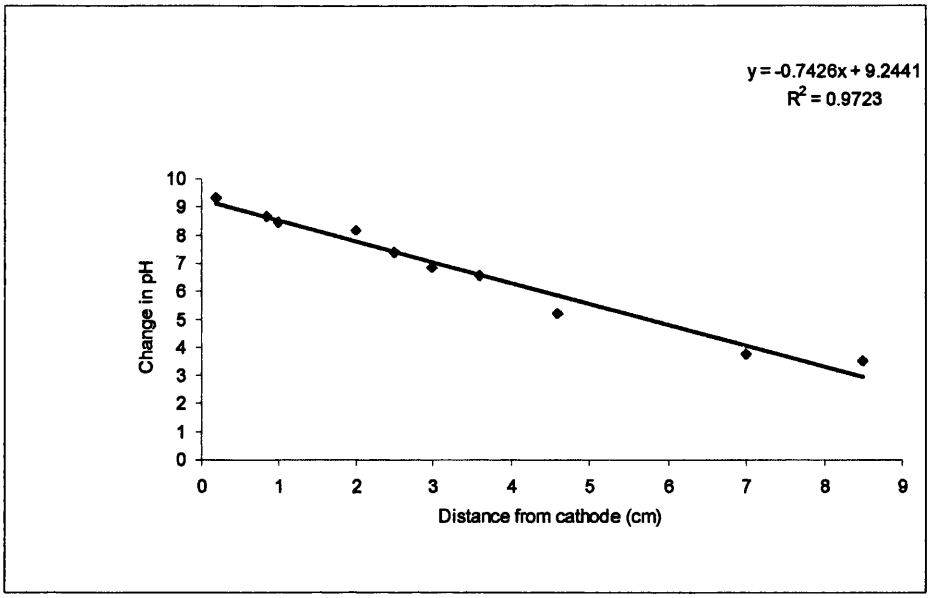


**Figure 2.4 Determination of pH gradient using the broad pI calibration kit on an Ampholine PAGplate, polyacrylamide gel.**

## 5 OXIDATIVE BURST: $H_2O_2$ ESTIMATION

The method of Glazener et al. (1991) was used to estimate $H_2O_2$-scavenging by luminol-dependent chemiluminescence in cassava suspension cells challenged with elicitor(s).

Suspension cells of cassava were collected by centrifuging at 200 x g for 5 min in a bench centrifuge at room temperature from cultures and washed with 150 ml of assay medium containing 0.5 mM of $CaCl_2$; 0.5 mM $K_2SO_4$; 175 mM mannitol; and 0.5 mM MES (2-[N-Morpholino] ethanesulfonic acid) buffer was adjusted to pH 5.8-6.0 with NaOH. The collected cells were suspended at 0.05 g/ml of assay medium. Large cell aggregates were removed by filtering the cell

suspension through a plastic mesh of 5 μM pore size. For all assays, 20 ml samples of collected cell suspensions were transferred to 50 ml sterile jars and pre-incubated for 4 h on a rotary shaker at 27°C and 180 rpm.

### 5.1 $H_2O_2$ SCAVENGING ASSAY

The luminol-dependent chemiluminescence was measured with a LKB 1251 luminometer equipped with automatic sample dispensers and an IBM XT computer for data collection. The luminol (N- (4-aminobutyl)-N-ethylisoluminol) solution used in the experiments was 3.5 mg/ml in DMSO. 20 ml of cell suspensions was transferred to 50 ml sterile jars and pre-incubated for 2-3 h in a rotary shaker at 27°C and 180 rpm. The elicitor was then added and $H_2O_2$ concentration measured every 30 sec. After the addition of elicitors, 40 μl of cassava suspension cells were dispensed with 100 μl of 50 mM pH 9.0 CHES buffer (2-[N-cyclohexylamino] ethanesulfonic acid) into a polystyrene cuvette for the assay. The mixture of horseradish peroxidase (type II) 1 mg/ml final concentration with luminol were kept at 25°C in a dark sterile tube during the time of the experiment and 60 μl were automatically dispensed by the machine to give final concentrations of 0.03 mg/ml.

# 6 MOLECULAR METHODS

## 6.1 DNA EXTRACTION

### 6.1.1 Genomic DNA

Genomic DNA was extracted from cassava leaves according to (Dellaporta et al. 1983). Young leaf tissue was collected and immediately frozen in liquid nitrogen and stored at – 70°C until required; then tissue was ground with liquid nitrogen. Four grams of powder was placed in an Oakridge tube and mixed with 15 ml of 65°C warm Dellaporta extraction buffer[5], followed by the addition of 1 ml 20% sodium dodecyl sulphate (SDS). This suspension was incubated in a shaking water bath at 65°C for 10 min and every 2 min the tubes were inverted twice before returning to the water bath. After this time, 5 ml of 5 M potassium acetate was added and the tubes were mixed vigorously. Tubes were then incubated on ice for 40-50 min before centrifugation at 13,000 x g for 20 min at 4°C. The supernatant was filtered through miracloth into a new Oakridge tube containing 10 ml of isopropanol precooled at –20°C, then mixed slowly and incubated at –20°C for a minimum of 2 h (or overnight) then centrifuged at 13,000 x g for 15 min at 4°C. The supernatant was discarded and the pellet was dried at room temperature. The pellet was redissolved in 700 µl of SDW and transferred to a microcentrifuge tube, and centrifuged at 12,000 x g for 10 min at room temperature. The supernatant was transferred to

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[5] Dellaporta extraction buffer

100 mM of Tris-HCl pH 8,0 was mixed with 50 mM of EDTA and 500 mM of sodium chloride. This solution was autoclaved and before use, 0.07% β-mercaptoethanol and 1% polyvinylpyrrolidone (PVP) was added.

a new microcentrifuge tube containing RNase at a final concentration of 10 μg/ml, and incubated in a water bath at 37°C for 30 min. 75 μl of 3 M sodium acetate and 500 μl of isopropanol precooled to –20°C was added to the microcentrifuge tube, and the tubes incubated at –20°C minimum 2 h or overnight. The tubes were centrifuged at 13,000 x g for 15 min at 4°C. The supernatant was discarded and 500 μl of 70% ethanol was added to the pellet. The tubes were centrifuged at 13,000 x g for 15 min at 4°C. The supernatant was discarded and 500 μl of 70% ethanol was added to the pellet. After the last wash the supernatant was discarded and the pellet was vacuum dried for 10-15 min. Finally the pellet was dissolved in SDW and stored at – 20°C until required. Before use the integrity of the DNA was checked on a 0.8% agarose gel.

### 6.1.2 Plasmid DNA

Minipreparation of plasmid DNA was obtained by the alkaline lysis method (Sambrook, Fritsch, & Maniatis 1989). A single bacterial colony was picked from a Luria Broth[6] (LB) plate and inoculated in 2 ml of LB liquid supplemented with the antibiotic ampicillin (50 μg/ml). The culture was grown overnight at 150 rpm on a shaker at 37°C. 1.5 ml of this culture was transferred

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[6] Luria Broth medium
10 g of bacto tryptone was mixed with 5 g of bacto yeast extract and 10 g of sodium chloride. The pH was adjusted to 7.0 before adding agar 1.5% (w/v) and made up to 1 l with SDW. The solution was sterilised by autoclaving.

to a sterile microfuge tube and then centrifuged at 12,000 x g for 30 sec at 4°C. The supernatant was removed and the bacterial pellet was allowed to air dry before the tube was placed on ice. The pellet was re-suspended by vigorous vortexing in 100 µl of Solution I[7], and 200 µl of Solution II was then added to the tube. After inverting the tube several times 150 µl of Solution III was added to the tube and the solution was mixed by gently vortexing the tubes in an inverted position. Tubes were stored on ice for 5 min before centrifugation at 12,000 x g for 5 min at 4°C. The supernatant was transferred to a new microfuge tube and mixed with 2 vol. of ethanol at room temperature, then left to stand for 5 min. Plasmid DNA was recovered by centrifugation at 12,000 x g for 5 min at 4°C and the pellet was washed with 70% ethanol at 4°C. Finally, the pellet was recovered by centrifugation at 12,000 x g for 5 min at 4°C and was dried by 5 min vacuum drying and re-suspended in 50-100 µl of SDW containing DNase free pancreatic RNase at a final concentration of 20 µg/ml and stored at –20°C.

## 6.2 POLYMERASE CHAIN REACTION

A polymerase chain reaction (PCR) technique was used with cassava genomic DNA extracted from leaves. A standard protocol for PCR amplifications was used with the reagents in a MJ thermocycler PTC-100, using

[7]Solution I
50 mM glucose mixed with 25 mM Tris-Cl pH 8.0 and 10 mM EDTA pH 8.0, made up to 100 ml with SDW and autoclaved. Stored at 4 °C.
Solution II
0.2 N NaOH mixed with 1% of SDS. This solution was freshly prepared.
Solution III
60 ml of 5 M potassium acetate mixed with 11.5 ml of glacial acetic acid and 28.5 ml of SDW.

the parameters summarised in **(Tables 2.0 and 2.1)**. Taq polymerase was obtained from (Appligene oncor) and dNTP's were from (Pharmacia).

| | |
|---|---|
| **Genomic DNA** | 10 ng |
| **10X reaction buffer** | 1X |
| **Each primer** | 1 μM |
| **dNTPs*** | 0.2 mM each |
| **Taq polymerase** | 1 U |
| **$Mg^{2+}$** | 1.5 mM |
| **SDW** | To 25 μl |

**Table 2.0 Reagents for standard PCR with degenerate primers.**

**dNTPs**: dACTP, dGTP, dCTP, and dTTP nucleotides.

| | | |
|---|---|---|
| **Denaturing** | 93 °C | 30 sec |
| **Annealing** | 93 °C | 5 sec |
| **(35 cycles)** | 55 °C | 1 min |
| | 72 °C | 3 min |
| **Extension** | 72 °C | 10 min |
| **Hold** | 4 °C | 1 h |

**Table 2.1 Standard PCR protocol in the MJ PTC-100 thermocycler.**

### 6.2.1 Degenerate Primers

Twelve degenerate primers were constructed based on a multiple sequence alignment analysis using CLUSTAL-W software (Higgins DG et al. 1996) **(Table 2.2)**.

| Primer | No. Bases | Sequence |
|---|---|---|
| 139f | 23 | TT(C/T)T(T/A)(T/C)(G/C)T(G/T)AATGG(A/C/G/T)A(A/C/G/T)(A/C/G/T)AAGGT |
| 459f | 27 | GG(A/C/G/T)TT(C/T)TG(C/T)AC(A/C/G/T)CC(C/G/T)GG(A/C)AT(C/T)GT(C/G/T)ATG |
| 557f | 20 | CG(G/C)TG(C/T)CA(A/C/G/T)GG(A/C)TA(C/T)(C/T)GACC |
| 1563f | 14 | T(A/T)TGG(C/T)GG(A/T)ATGGC |
| 2176f | 18 | TGA(C/T)GAG(A/C/G)(A/C/T)(A/C/T)(G/T)TCTTTGC |
| 2959f | 20 | AA(A/C/G/T)GC(A/C)TTCCG(G/T)GG(A/C)TTTGG |
| 3768f | 21 | CAGA(C/T)AT(C/T)(A/G)(A/T)(A/C/G/T)ATGGA(C/T)(A/G)GGC |
| 1466r | 22 | T(C/T)AC(A/C/G/T)(T/A)T(A/G/T)GC(T/A)ATGTCATC(A/C/G/T)TC |
| 1469r | 18 | T(C/T)AC(A/C/G)T(A/G/T)GC(A/T)ATGTCATC |
| 2943r | 28 | C(C/T)TG(A/G/T)GG(A/C/G/T)CC(A/C/T)CCAAA(G/T)CC(A/C)CGGAA(G/T)GC |
| 3745r | 23 | GCC(A/T)A(C/T)(A/G)TCCAT(A/C/G/T)(A/T)(C/T)(A/G)AT(A/G)TCTG |
| 4003r | 21 | GAA(A/C/G/T)AG(A/C/G/T)GG(A/C/G/T)GG(C/T)TC(A/C/G/T)CC(A/C/G/T)AA |

**Table 2.2 Degenerate primers for xanthine oxidase gene cloning.**

**f**: Forward; **r**: Reverse

## 6.3. ELECTROPHORESIS OF DNA

DNA samples were separated by agarose gel electrophoresis under conditions of voltage gradient, time and agarose concentration that were appropriate for resolving the molecules of interest (Sambrook, Fritsch, & Maniatis 1989). 1XTAE buffer[8] was used for electrophoresis. DNA molecular weights markers, 100 bp ladder (Pharmacia) or *Hin*dIII cut lambda (λ) DNA (New England Biolabs) were used as appropriate. DNA samples were mixed with 0.2 volumes of loading buffer before loading on the gel (Sambrook, Fritsch, & Maniatis 1989). DNA bands were visually quantified by comparison to known amounts of (λ) DNA. Gels were stained in ethidium bromide (0.5 μg/ml) for 20 min and photographed with a UVP white/UV transilluminator and digital graphics printer (UPD86OE Sony).

### 6.3.1 Recovery of DNA from Agarose Gels

PCR products or vector insert DNA bands of interest were isolated from the agarose gels using the Sephaglass kit (Pharmacia Biotech) according to the manufacturer's instructions.

[8] Tris-Acetate (TAE) 1X Buffer
242 g of Tris base was mixed with 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) and made up to one litre with SDW.
Gel loading buffer
0.25% bromophenol blue (w/v) was mixed with 40% sucrose (w/v) and made up with SDW and store at room temperature.

## 6.4 PREPARATION OF *E. coli* COMPETENT CELLS

Competent cells were obtained using JM101 *E. coli* strain by the calcium chloride method (Sambrook, Fritsch, & Maniatis 1989). A single colony was picked from a freshly grown LB plate and inoculated to 100 ml SOB[9] medium. The culture was grown for 3 h ($OD_{600}$ = 0.5 ~ 3.18 x $10^8$ cells/ml). This culture was transferred to on Oakridge tube and the tube was stored on ice for 10 min. The cells were recovered by centrifugation at 4,000 x g for 10 min at 4°C. The supernatant was poured off, the pellet re-suspended in 10 ml ice cold 0.1M $CaCl_2$ and subsequently, the tube was centrifuged at 4,000 x g for 10 min at 4°C. The supernatant was decarted and, the pellet re-suspended in 2 ml ice cold 0.1 M $CaCl_2$. The competent cells were used immediately.

## 6.5 LIGATION OF PCR PRODUCTS TO A PLASMID VECTOR

pGEM-T vector (Promega) was used according to manufacturer's instructions. A standard protocol for ligation was followed **(Table 2.3)**. After mixing all the reagents by pipetting, the microfuge tube was incubated overnight at 12°C.

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[9] SOB medium
20 g of bacto-tryptone were mixed with 5 g of yeast extract and 0.5 g of sodium chloride; these reagents were dissolved into 950 ml of SDW and 10 ml of 250 mM potassium chloride was added. The pH was adjusted to 7.0 and the volume made up to 1 l with SDW before autoclaving. After autoclaving 5 ml of 2 M magnesium dichloride was added.

## 6.6 TRANSFORMATION OF COMPETENT *E. coli* CELLS

200 µl of JM101 competent *E. coli* cells were mixed the ligated DNA's and gently stirred before storage on ice for 30 min. The tube was placed in a water bath at 42°C for exactly 90 sec and then returned to ice for another 2 min. 800 µl of SOC[10] medium was added and the tubes were incubated at 37°C at 50 rpm for 1 h. In order to allow for blue/white colour screening of transformant colonies 10 µl of 0.1 M IPTG and 44 µl X-Gal (20 mg/ml in dimethylformamide) was spread over the surface of a LB agar plate supplemented with the antibiotic ampicillin (100 µg/ml final concentration). 200 µl of the transformed cells were spread onto the LB plate and allowed to dry for 1 h before being incubated at 37°C overnight.

| | Standard reaction | Positive control |
|---|---|---|
| **T4 DNA Ligase 10 X buffer** | 1.6 µl | 1.6 µl |
| **pGEM-T vector (5 ng/µl)** | 1 µl | 1 µl |
| **PCR product (fragment )** | X µl | - |
| **Control insert DNA (542 bp/12 µl)** | - | 2µl |
| **T4 DNA Ligase (3 Weiss units/µl)** | 1 µl | 1 µl |
| **SDW to a final volume of** | 16 µl | 16 µl |

**Table 2.3 Reagents for standard 16 µl ligation with pGEM T vector.**

[10] SOC medium
20 g of bacto-tryptone, 5 g of yeast extract, and 0.5 g of sodium chloride were dissolved in 950 ml of SDW and 10 ml of 250 mM potassium chloride. The pH was adjusted to 7.0, the volume made up to 1 l with SDW and the medium autoclaved. After autoclaving, 5 ml of 2 M magnesium dichloride and 20 ml of filter sterile 1 M glucose were added.

## 6.7 ANALYSIS OF TRANSFORMANTS

*E. coli* harbouring recombinant plasmids will produce white colonies, while those with non-recombinant plasmids should be blue. White colonies were selected and grown in LB medium with ampicillin (50 μg/ml final concentration) followed by miniprep alkali lysis according to (Sambrook, Fritsch, & Maniatis 1989).

## 6.8 RESTRICTION DIGESTION OF DNA

DNA solutions were digested with restriction enzymes according to the manufacturer's instructions (Kramel Biotechnologies, New England Biolabs, Bioline or Advance Biotechnologies). A digestion was performed with 1 μg of DNA in 2 μl of appropriate enzyme buffer (10X), 1 μl of digestion enzyme and made up to 20 μl of SDW. Reactions were incubated at 37°C for 3 h and then kept on ice or frozen until needed.

## 6.9 GENE EXPRESSION

### 6.9.1 RNA Extraction

Total RNA was extracted from cassava suspension cells, following the procedure described by the SV total RNA isolation system kit (Promega). 0.5 g of cassava cells were mixed with 200 μl of SV RNA lysis buffer. Then 500 μl SV RNA dilution buffer was added, the tube was inverted 3-4 times and placed at 70°C for exactly 3 min. The sample was then centrifuged at 12,000 x g for 10 min at room temperature. The clear lysate supernatant solution was transferred

to a new microfuge tube and 500 µl of 95% ethanol was added, then the solution was mixed by pipetting 3 - 4 times. The vacuum adapter was attached to the port of the vacuum manifold, SV RNA spin basket and the collection tube, the clear supernatant was applied to the centre of the column and the vacuum was applied to the system. Then, 900 µl of SV RNA wash solution was added to the column and the vacuum was applied again to the system. For each isolation, DNase mix was prepared by combining: 40 µl of yellow core buffer, 5 µl of 0.09 M $MnCl_2$, 5 µl of DNase I enzyme, and this mix was applied to the centre of each column. The column was incubated at room temperature for 15 min. Once the incubation was complete, 200 µl of SV DNase stop solution was applied to the column. Before recovering the RNA, the column was washed twice with 900 µl of SV RNA wash solution. Finally the RNA sample was eluted by applying 50 µl of nuclease-free water to the column and centrifuging for 1 min at 12,000 x g at room temperature. The samples were stored at –70°C.

### 6.9.2 Determination of RNA Concentration

The amount of RNA was determined spectophotometrically based on the assumption that 1 $OD_{260nm}$ = 40 µg ml/l. The average RNA yield using SV minipreps was 100 µg/g cells. All samples were adjusted to 1 µg/µl RNA concentration. To ensure that there was the same amount of RNA for Northern analysis, aliquots of RNA were mixed with 3.5 µl of ethidium bromide (from a stock at 10 mg/ml made in RNase-free water) and then separated on a 0.8% agarose gel at 5 volts/cm for 1 h.

### 6.9.3 RNA Markers

RNA markers were obtained from New England Biolabs. Six μl of marker was mixed with 4 μl 5X MOPS, 2 μl formaldehyde (37%), and 8 μl distilled RNase-free water. The solution was denatured for 5 min at 70 °C and the sample was chilled on ice. Before loading the marker into the gel, 4 μl of loading buffer with ethidium bromide (0.05 μg/μl) was added to the markers. After the overnight transfer onto a nylon membrane, the markers were cut from the nylon membrane and stained with methylene blue solution[11] (Herrin & Schmidt 1988).

### 6.9.4 Northern Blots

#### 6.9.4.1 Electrophoresis of RNA

10 μg of cassava RNA sample was made up to 20 μl with RNA-loading mixture[12] according of (Sambrook, Fritsch, & Maniatis 1989). The RNA was

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[11] Methylene blue solution
0.02% of methylene blue (Fisher) was mixed in 0.3M sodium acetate (pH 5.5) and stored at room temperature. Fix the RNA marker by stain at least 24 h. Destain the RNA marker with 20% ethanol, until background is clear.
RNA loading buffer
5 ml glycerol (50% v/v with DEPC-SDW), 20 μl EDTA (1 mM; pH 8.0), 25 μl bromophenol blue (0.25% v/v with DEPC-SDW), 25 μl xylene crystal (0.25% v/v with DEPC-SDW) were mixed and made up to 10 ml with DEPC-SDW.

[12] RNA loading mixture
2.2 M formaldehyde, 1X MOPS buffer, and DEPC treated-SDW was made up to 20 μl before pipetting into the agarose gel.
5X MOPS buffer
0.2 M MOPS (pH7.0), 0.05 M sodium acetate, 0.005M EDTA (pH 8.0). The preparation was dissolved in DEPC-SDW and autoclaved.
DEPC-SDW STOCK
1% dimethylpyrocarbonate (DEPC) was dissolved in a 50% ethanol: SDW. 10% (v/v) was mixed with SDW and allowed to stand for 1 hour before autoclaving.
1.5% formaldehyde gel
0.48 g of agarose was dissolved in 26.5 ml DEPC-SDW. This preparation was mixed with 8.25 ml of 5X MOPS buffer, and 7.5 ml of formaldehyde.

denatured for 15 min at 65°C and the tubes were transferred to ice for 5 min. 4 µl of RNA loading buffer containing ethidium bromide at a final concentration of 0.005 µg/µl in RNase-free water (DEPC-SDW) was added to the samples before running. 6 µl of RNA marker ladder (New England Biolabs) was mixed with 4 µl of 5X MOPS buffer 2 µl of formaldehyde, and 8 µl of DEPC-SDW, before denaturation for 5 min at 70°C and transfer to ice for 3-5 min. The RNA samples were separated on a 1.5% formaldehyde gel. The gel was run at 5 V/cm for 4-5 h in 60 ml of 5X MOPS buffer, 54 ml formaldehyde and 186 ml DEPC-water. Afterwards the gel was washed twice for 10 min in DEPC-SDW. The success of the separation and RNA concentration was checked on a transilluminator at $254_{nm}$.

#### 6.9.4.2 RNA-Blotting

After electrophoresis, the DEPC-SDW washed gel was placed on a Northern blot apparatus. A glass tray was cleaned with DEPC-SDW and filled with 20XSSC buffer[13]. A plastic tray cleaned with DEPC-SDW was placed on the glass tray and 2 pieces of Whatman No.1 paper soaked with 20XSSC buffer was placed on the top of the plastic tray. The gel was then placed inverted on the wet paper and overlaid with a piece of nylon membrane Hybond $N^+$ (Amersham).

[13] 20XSSC buffer
175.4 g of sodium chloride were mixed with 88.2 g of sodium citrate made up to 1 l with SDW. Finally the pH was adjusted to (pH 7.2) with 10 N NaOH, and autoclaved.
2XSSC buffer
100 ml of 20XSSC was mixed with 900 ml of DEPC-SDW and autoclaved.

Two pieces of Whatman No.1 paper soaked with 2 XSSC buffer was placed on the top of nylon membrane. Absorbent tissue paper was placed above the pieces of filter paper. Finally, a glass plate was placed on the top and a weight of ~ 500 g was added to the plate. The transfer of RNA from the gel onto the positively charged nylon membrane was performed overnight. After blotting the membrane was dried for 20 min at room temperature and the RNA fixed on the membrane with an UV cross-linker ($254_{nm}$, Amersham) for 3 min, enveloped with Saran wrap plastic, and stored at – 20°C until use.

#### 6.9.4.3 Preparation of DNA Probes

Homologous and heterologous probes were used (**Table 2.4**). The homologous probes were isolated from a cDNA library constructed by Y. Han (Beeching et al. 1997). The cDNA library was constructed in λgt10 using the Amersham Rapid cDNA-cloning module, from total RNA isolated from cassava cultivar MNGA 1 storage roots 48 h after harvest. The alkali lysis miniprep was performed to obtain plasmid DNA (Sambrook, Fritsch, & Maniatis 1989). Plasmid DNA was digested with the appropriate restriction enzyme according to the manufacturer's instructions (New England Biolabs). Samples were separated agarose gel electrophoresis, and the insert bands were cut from the gel and purified using the QIAEX II Gel extraction kit (QIAGEN), according to manufacturer's instructions.

| Homologous probes | Name | Restriction enzyme to release insert and expected insert size | Reference or source |
|---|---|---|---|
| Catalase cDNA | MecCAT1 | *Eco*RI 1.3 kb | (Reilly et al. in press) |
| HRGP cDNA | MecHRGP1 | *Eco*RI 1.6 kb | (Han et al. in press) |
| Glucanase cDNA | MecGlu1 | *Eco*RI 1.2 kb | (Han et al. 2000) |
| PAL cDNA | MecPAL1a | *Eco*RI 1.5 kb | (Beeching et al. 1997) |
| POD cDNA | MecPOD1 | *Eco*RI ~ 600 bp | K. Reilly, unpublished |
| House-keeping gene | 18SrDNA | *Eco*RI/*Hind*III ~ 600 bp | (Reilly et al. in press) |
| **Heterologous probe** | | | |
| Tobacco pTCAD19 | CAD | *Eco*RI 1.4 kb | (Knight et al. 1992) |

**Table 2.4 Homologous and heterologous probes used for gene expression.**

**CAD**: Cinnamyl alcohol dehydrogenase; **CAT**: Catalase; **GLU**: Glucanase; **HRGP**: Hydroxyproline-rich glycoprotein; **PAL**: Phenylalanine ammonia-lyase; **POD**: Peroxidase.

#### 6.9.4.4 Oligolabelling with $^{32}P$

The Pharmacia Biotechnology labelling kit was used. 25 ng of DNA probe was dissolved in DEPC-SDW and after denaturing at 100°C for 3 min, the sample was placed on ice for 3-5 min. The sample was mixed with the following solutions: 10 µl of reagent mix ([pd(N)$_6$/dNTPs/buffer]), 2.5 µl of [$\alpha$-$^{32}$-P] dCTP (50 µCi), 49 µl distilled sterile water, and 1 µl of FPLC pure Klenow fragment (1U/µl) (Pharmacia Biotech). This solution was mixed gently and incubated at 37 °C for 60 min. Once the incubation had finished at 37°C, the labelled-probe was passed through a Nick™ Sephadex G-50 column to remove

unincorporated nucleotides (Pharmacia Biotech), using spun column buffer[14], following the manufacturer's instructions. Afterwards the labelled-probe was denatured for 3 min at 100°C and placed on ice for 3 min before use.

#### 6.9.4.5 Northern Hybridisation

Pre-hybridisation and hybridisation of membranes was performed in tubes rotated and incubated in a Hybaid hybridisation oven (HB-OV-1). Membranes for Northern blotting were placed in a glass hybridisation tube with 15 ml of pre-hybridisation solution[15]. For homologous probes, membranes were pre-hybridised at 65°C for 1 hour. With non-homologous probes, the temperature was 60 °C.

The pre-hybridisation buffer was replaced with fresh 15 ml of pre-hybridisation solution. [$^{32}$P]-labelled probe was then added to the glass tube and, the nylon membrane was hybridised overnight at 65°C (homologous probes), and 60°C (heterologous probes).

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[14] Spun column buffer
20 mM of Tris-HCl (pH 7.5) was mixed with 20 mM of sodium chloride and 2 mM of EDTA and 0.25% of SDS before autoclaved.

[15] Pre-hybridisation solution
0.5 M sodium phosphate buffer (pH 7.2), 1% skimmed milk (w/v), and 7% sodium dodecylsulphate (SDS) (w/v) were made up to 1 l of DEPC-SDW. The pH was adjusted to 7.2 with 10 N NaOH.

For high stringency conditions with homologous probes, the nylon membrane was first rinsed with 1XSSC, 0.1% SDS[16] three times for 2-3 min at room temperature. This was followed by a wash in 0.2XSSC, 0.2%SDS for 20 min at 65°C until the reading on the Mini-Monitor G-M (Mini Instruments Ltd. Essex, U.K.) reached ~ 15 cpi. For heterologous probes, low stringency washing conditions were used. The membrane was rinsed with 1XSSC, 0.1% SDS three times for 2-3 min at room temperature. This was followed by a wash in 2XSSC, 0.2%SDS for 20 min at 60°C until the reading on the Mini-Monitor G-M reached approximately 15 cpi.

#### 6.9.4.6 Autoradiography

After washing, the membrane was removed and wrapped in Saran-Wrap plastic film. Autoradiography was obtained by exposing the membrane on X-ray cassette with intensifier screens to a pre-flashed X-ray film (Kodak X-OMAT AR 18 x 24 cm) at –70 °C. The film was pre-flashed by exposure to a stroboscope, which had been calibrated to give an increase in absorbance at $545_{nm}$ of 0.15 above that of unflashed films. The X-ray film was developed after a suitable time exposure time in a developing machine (X-OMAT 2X Processor).

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[16] 1XSSC, 0.1% SDS
50 ml of 20XSSC stock buffer were mixed with 5 ml of 20% SDS stock solution and made up to 1 l with DEPC-SDW.
0.2XSSC, 0.2% SDS
10 ml of 20XSSC stock buffer were mixed with 10 ml of 20% SDS stock solution and made up to 1 l with DEPC-SDW.

#### 6.9.4.7 Equal Loading Estimation

In order to demonstrate equal loading, blots were stripped and re-probed using a cassava cDNA clone for the 18SrRNA subunit for stripping blots. The nylon membrane was immersed into boiling water with 1% SDS and allowed to cool down. This step was repeated until the reading on the Mini-Monitor G-M reached approximately 2 cpi.

# 7 CHROMATOGRAPHIC ANALYSIS

## 7.1 EXTRACTION OF PHENOLS FROM CASSAVA SUSPENSION CELLS

Cassava cell suspensions were ground with nitrogen liquid, and dissolved in 95% methanol (1:5 w/v) (HPLC grade, Rathburn Chemicals Limited, Walkerburn, Scotland, U.K.). This solution was homogenised for 3 min in an Ultraturrax (IKA), following by incubation at 48°C for 1 hour. The sample was centrifuged for 5 min at 3,000 x g in a bench centrifuge. The supernatant was recovered and the solvent evaporated (BÜCHI, Rotavapor-R-4) at 40°C until the pellet was dry.

The following step was repeated three times. The pellet was resuspended in 1:1 ethyl acetate: water (pH 8.0), and after shaking, the ethyl acetate fraction was collected. This solvent was completely evaporated and the final pellet was resuspended in 1 ml of methanol. After the third extraction the final extract was filtered through a syringe filter (Hplc technology, pfte 0.22 μm, 1.3 mm), and transferred into a brown glass sample vial and stored at –20°C until use. In the case of the cell suspension supernatant, 15 ml of medium was

completely dried in a speed-vacuum concentrator (Savant Instruments; Farmingdale, N.Y., U.S.A.), and stored at –20°C until use. The pellet was resuspended in 200 µl of methanol and filtered through a syringe filter (Hplc technology, pfte 0.22 µm, 1.3 mm) before use.

### 7.2 EXTRACTION OF PHENOLS FROM CASSAVA LEAVES

Cassava leaves discs were collected after elicitation and grounded with liquid nitrogen, and extracted with methanol (1:10 w/v) (HPLC grade, Rathburn Chemicals Limited, Walkerburn, Scotland, U.K.). This suspension was homogenised for 3 min in an Ultra turrax (IKA), following by incubation at 48°C for 1 hour. The sample was centrifuged for 5 min at 3,000 x g, then filtered through Whatman No.1 paper filter paper. The supernatant was recovered and the solvent was evaporated at 40°C until dry. The dried residue was dissolved in 200 µl methanol. In order to remove chlorophyll from the sample 200 µl of water was added. The sample was put on ice until the chlorophyll precipitated out after about 4 h, when the sample was centrifuged for 2 min at 4°C at 10,000 x g in a bench centrifuge. The supernatant was removed and transferred to microfuge tube and placed at –20 °C for 1 hour. If after 1 h there was a pellet, the tube was centrifuged for a further 2 min at 4°C at 10,000 x g . The final extract was passed through a syringe filter (Hplc technology, pfte 0.22 µm, 1.3 mm), and transferred into a brown glass sample vial and stored at -20°C until use. The extract was then subjected to both TLC and HPLC analysis. For quantification analysis, external standards were used, these reference

compounds were all from Sigma except kaempferol 3-O-rutinoside, which was from Roth Ltd., Germany.

### 7.3 HPLC SEPARATION OF PHENOLICS

A reverse phase high performance liquid chromatography (RP-HPLC) method was used to analyse phenolic compounds from the cassava cell suspensions, culture media and from the cassava leaves. From the extracts obtained above, 5 µl of extract for leaf extracts and 20 µl for cells and media extracts were injected. The parameters for the HPLC-machine were as shown in **(Table 2.5).**

A linear gradient system of solvent A, phosphoric acid ($H_3PO_4$) pH 2.6 and methanol (9:1, v/v) (0.5% aqueous) and solvent B, acetonitrile ($CH_3CN$), from 0 to 100% in 30 min, and a final wash for 5 min with 100% acetonitrile was used **(Table 2.6)**.

### 7.4 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) was used, by using HPTL Silica gel 60 $F_{254}$ and silica gel 60 $F_{254}$. According to the plate size a base line was pencilled 1.5-3 cm from the base of the pre-cleaned TLC plate (the TLC plate was washed in a solvent run and left to dry before use). 40-100 µl of the samples were applied either as spots or as lines (each sample was separated by 0.5-1 cm) and dried in a hot air stream. The solvent mixture for the TLC plates was

chloroform: ethyl acetate: methanol (2:2:1). The silica plate was immersed to a depth of 0.5-2 cm in a solvent mixture and the solvent front was allowed to migrate until the samples reached approximately 75% of the TLC plate. The silica plate was allowed to dry completely, then separated compounds were detected either by auto-fluorescence, (extinction of fluorescence at $254_{nm}$ or $366_{nm}$ CAMAG, UV lamp) or by analytical staining, and photographed.

| | |
|---|---|
| **Stationary phase and column dimensions** | Dionex. GROM-Sil 120 ODS (2) 5μm particle size 250 x 4.6 mm (GROM, Germany) |
| **Pump** | Dionex P580 |
| **Injector** | Rheodyne, manual with 20μl loop |
| **Detector** | UVD170S Simultaneously at 215, 260, 280 and 350 |
| **Detection Wavelength** | 215, 260, 280 and 350 nm simultaneously |
| **Control and evaluation software** | Dionex Chromeleon |

**Table 2.5 Parameters for HPLC equipment**

| Time | CH3CN(%) | Flow Rate (ml/min) |
|---|---|---|
| 0-5 | 0 | 0.8 |
| 5-40 | 70 | 0.8 |
| 40-42 | 80 | 0.8 |
| 42-47 | 100 | 0.8 |
| 47-50 | 80 | 0.8 |
| 50-55 | 0 | 0.8 |

**Table 2.6 Parameters for the gradient system**

## 7.5 STAINING PROTOCOLS

The following staining procedures of the TLC plate was used (Krebs et al. 1990). After spraying the TLC plates with the stain solution, the TLC plate was heated at 100 - 105°C until the spots attained maximum colour intensity.

### 7.5.1 Folin-Ciocalteu Reagent

10 g of sodium One ml of concentrated sulphuric acid was added to a solution of 0.5 ml anisaldehyde in 50 ml acetic acid. This reagent detected phenols, terpenes, sugars and steroids giving violet, blue, red, grey or green bands, depending on the compound.

### 7.5.2 DPPH Reagent

The silica plate was immersed for 1 sec in a solution of 5 mg of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical dissolved in methanol. This reagent detected antioxidant metabolites (Nakamura et al. 1991; Takao et al. 1994). Clear bands on a pink-purple background reveal the presence of antioxidant metabolites.

### 7.5.3 Ehrlich's Reagent

1% *p*-dimethylaminobenzaldehyde in ethanol acidified with concentrated HCl, was used (Geissmann & Griffin 1971). This reagent detected amines and terpenoids.

7.5.4 Neu's Reagent

1 g diphenylboric acid-2-aminoehtyl ester dissolved in 100 ml methanol. This reagent detected flavonoids, carbohydrates, anthocyanidines and hydroxy- and methoxycinnaminc acids.

# 8 BIOASSAYS FOR ANTIFUNGAL OR ANTIBACTERIAL ACTIVITY

## 8.1 BACTERIAL ISOLATES

50 ml NYGB[17] were inoculated with *Xam* (strain 2967) and grown overnight at 200 x g at 28°C. The bacteria were harvested by centrifugation at 3000 x g for 15 minutes and the pellet were washed twice with SDW. The pellet was resuspended in SDW and the $OD_{600}$ determined using NTGB as a blank, in a CECIL Spectrophotometer. The bacterial suspension was diluted to give a range of absorbance values at 600nm between 0 and 1 OD units. Serial dilutions were performed until this bacterial suspension reached $10^8$ cfu/ml.

## 8.2 FUNGAL CULTURES

*Fusarium solani* and *Fusarium oxysporum* were cultured for three days on potato dextrose agar plates (PDA)[1]. *Trichoderma harzianum* was cultured for four days on malt agar plates[17]. Five ml of a sterile pH 6 solution of 15%

[17] Culture media (NYGB)
5 g of bacteriological peptone (Oxoid), 3 g of yeast extract (Oxoid), 20 g glycerol were dissolved and made up to 1 l with SDW prior to autoclaving.
Malt agar media
20 g of malt extract (Oxoid) and 18 g of agar were dissolved in 1 l SDW prior to autoclaving.

Czapek Dox liquid medium in SDW was poured on to each plate and the mycelium was mixed using a sterile toothpick to suspend the spores. The suspension was recovered and filtered through 3 layers of sterile muslin to remove fragments of mycelium and agar and the spore suspension was diluted to approximately 2.0 x $10^5$ spores/ml with more Czapek Dox solution. This conidia suspension was used for slide bioassays, and the same concentration of conidia suspension was used for TLC bioassays except that they were resuspended in FSM medium[18] instead.

## 8.3 ANTIFUNGAL BIOASSAYS

### 8.3.1 Antifungal Slide Bioassays

A slide bioassay technique was devised for the antifungal evaluation of four phenolic compounds (scopoletin, esculetin, ferulic acid and quercetin) to spore germination of *Trichoderma harzianum*, *Fusarium solani* and *Fusarium oxysporum*. 5 mg of each phenolic compound (scopoletin, esculetin, ferulic acid and quercetin) were dissolved in 5 ml of

[18] FSM medium
2 g of sodium nitrate, 1 g of potassium phosphate dihydrogen, 0.5 g of magnesium sulphate and 15 g of sucrose were dissolved in SDW and 20 mg of ferric sulphate, 100 mg of zinc sulphate, 2 mg of cupric sulphate and 2 mg of magnesium chloride were added, the solution were made up to 1 l with SDW prior to autoclaving.

absolute ethanol. Serial dilutions were performed to obtain solutions of the following final concentrations: 10, 50, 100, 200, 500, and 1000 μg/ml. Teflon™ microscopeslides (BDH) divided into six circular areas (7mm diameter) (BDH, Poole, Dorset, U.K.) were used. The slides were washed twice in 70% ethanol, twice with SDW and then dried in an oven at 85°C. Once ready, 50μl of the phenolic solution was deposited in one of the six circular areas and allowed to evaporated and then, 25μl of the spore suspension were added. Each slide was transferred to an individual polyethylene box (20 x 20 cm) containing a moisturised sterile filter paper and incubated for 16 h at 25°C in the dark. After this time 10μl of 0.01% (w/v) cotton blue in lactic acid solution was added to stain conidia and germ tubes and to arrest further growth. A cover slip was placed onto the preparation and the slides were kept at 4°C in the dark until assessment. Two replicas of each compound concentration and control (solvent-only) were performed and assessed. Microscopic examination of spore germination was assessed by counting 100 randomly chosen spores in 3 fields of view of each of the two replica slides for each treatment. A spore was considered germinated if the germ tube was longer than half the diameter/length of the spore. Germ tube length was measured by means of an eyepiece graticule.

### 8.3.2 Antifungal Plate Bioassays

The effect on mycelial grown of four phenolic compounds was tested. PDA was used for *F. solani* and *F. oxysporum* and malt agar was used for *T. harzianum*. 200 μl of spore suspension was poured onto each test plate and

spread using a sterile plastic spreader. The plates were then incubated at 25°C in the dark for 18 h to allow an even lawn of mycelium to form. One control paper disc (Whatman) and two containing compound (of the same concentration) were placed around the edge of each of two replicate plates for each pathogen. The plates were sealed with Parafilm and incubated for 72 hours at 26°C. The plates were assessed visually for any inhibition zone below or around the paper discs before photographs were taken.

### 8.3.3 Antifungal TLC Bioassays

For TLC fungal bioassays the conidia suspension ($2x10^5$ conidia $ml^{-1}$) in FSM medium (Cooper & Wood 1975) was sprayed over TLC plates, in which cell extracts had been run and subsequently dried (Threlfall & Whitehead ). Then, the plates were placed in polyethylene boxes containing a moisturised sterile filter paper and incubated for 16 h at 26°C in the dark.

## 8.4 ANTIBACTERIAL BIOASSAYS

### 8.4.1 Antibacterial TLC bioassays

The antibacterial bioassays were performed following the same protocol as for fungi. *Xam* (strain 2967) was grown until ($10^8$ cfu./ml) in a bacterial culture media (NYGB) (Turner et al. 1984). An overlay medium[19] was prepared, and 25 ml of this medium was overlaid on the TLC plates in which the cells

[19] Overlay medium
NYGB media were supplemented with 14 g $l^{-1}$ technical agar (Oxoid) prior to autoclaving. Finally, 1mg/ml of 2,3,5-triphenyl tetrazolium chloride (TTC) and the bacteria suspension up to $10^8$ cfu./ml were added.

extract had been separated (Threlfall & Whitehead ). Then the plates were transferred on to polyethylene boxes lined with moistened paper and left at 26°C overnight before being photographed.

## 8.5 FUNGITOXIC EFFECT OF OXIDISED PHENOLICS

### 8.5.1 Preliminary Test

0.02 ml Peroxidase II was added to a well in a microtitre plate containing 0.2 ml phenolic substrate (prepared by dissolving 1 ml 1000 ppm phenolic solution in 1.25 ml 0.5 M phosphate buffer pH 6 and making up to 10 ml with SDW. 400 µl, 200 µl or 80 µl of 1% hydrogen peroxide was added to the well. Wells were also prepared containing 400 µl, 200 µl or 80 µl tyrosinase in place of peroxidase. Hydrogen peroxide was not added to these wells, as the enzyme does not require it as an oxygen donor. Wells containing buffer and peroxidase/tyrosinase only were also prepared. The plate was checked visually and then by plate-reader at $420_{nm}$ to ascertain whether the enzyme(s) had oxidised any of the phenolic compounds.

### 8.5.2 Fungitoxic Effects of Oxidised Phenolics

81 µl peroxidase/tyrosinase was added to a 2.5 ml microcentrifuge tubes containing 819 µl. As before, 400 µl, 200 µl or 80 µl 1% hydrogen peroxide in phosphate buffer pH 6 was added to the replicate. Replicates were also prepared containing tyrosinase in place of peroxidase. Again, hydrogen peroxide was not added to these replicates. Microcentrifuge tubes containing

buffer and peroxidase/tyrosinase only were also prepared. 100 µl of 2.0 x $10^5$ spores/ml *F. solani*, *F. oxysporum* and *T. harzianum* spore suspension was added to each microcentrifuge tube. After 30 minutes the samples were centrifuged for 15 minutes at 2000 x g. The supernatant was removed and the spores resuspended in 900 µl sterile solution of 15% Czapeck Dox pH 6. As described in section (8.3.1) 25 µl of spore suspension was put into each cavity Teflon™ microscope slides, and these were incubated at 26°C in the dark for 16 hours in a moist chamber. Then 10 µl of cotton blue in lactic acid was added and the slides assessed as described in section 8.3.1 **(Table 2.7)**.

| **Treatment Number** | **Added to spore suspension** |
|---|---|
| 1 | Esculetin + peroxidase + 0.1% $H_2O_2$ |
| 2 | Esculetin + peroxidase + 0.05% $H_2O_2$ |
| 3 | Esculetin + peroxidase + 0.02% $H_2O_2$ |
| 4 | Esculetin + tyrosinase |
| 5 | Scopoletin + peroxidase + 0.1% $H_2O_2$ |
| 6 | Scopoletin + peroxidase + 0.05% $H_2O_2$ |
| 7 | Scopoletin + peroxidase + 0.02% $H_2O_2$ |
| 8 | Buffer + peroxidase + 0.1% $H_2O_2$ |
| 9 | Buffer + peroxidase + 0.05% $H_2O_2$ |
| 10 | Buffer + peroxidase + 0.02% $H_2O_2$ |
| 11 | Buffer + peroxidase |
| 12 | Buffer + tyrosinase |
| 13 | Buffer only (control) |

**Table 2.7 Fungitoxic effects of oxidised phenolics treatments.**

### 8.5.3 Fungitoxicity of Cassava Cells and Leaf Extracts

Cassava cell suspensions challenged with yeast elicitor were prepared as described in section 7.1 and cassava leaf extracts as described in section 7.2. The dried extracts were re-dissolved in 200 μl 50% methanol : water. Serial dilutions were performed to reach concentrations of 5 g $ml^{-1}$, 0.5 g $ml^{-1}$ and 0.05 g/l. 50 μl of each were dried onto cavity Teflon™ microscope slides and these were assayed as described in section 8.3.1.

*Microsoft Excel* was used for calculated the six values obtained for each treatment. These were then converted into a mean as a percentage of the control. Percentage error were calculated using *Microsoft Minitab* was used to perfom an Analysis of variance test (ANOVA test), for each set of date that each treatment could be compared with every other treatment of a given experiment. The ANOVA tables are given in **Appendix 7**.

# CHAPTER 3

# ESTABLISHMENT OF CASSAVA CELL SUSPENSION CULTURES AND SELECTION OF ELICITOR OF DEFENCE RESPONSES

## 1 INTRODUCTION

A range of tissue culture techniques has traditionally been used with cassava in attempts to select healthy plants from diseased ones, and also to maintain germplasm collections. Initially, the most common methodologies were meristem and shoot-tip culture methods, later the introduction of cell and protoplast techniques also have been used in cassava as a tool for pathologists (Roca 1984).

The co-culture of cassava suspension cells with a pathogen causing bacterial blight (*Erwinia herbicola*) was reported by (Roach & Garnett 1986), who concluded that the pathogenic effect of this bacterium was due to the release of a toxic compound into the plant suspension culture medium. In the interaction between *X. axonopodis* pv *manihotis* (*Xam*) and cassava suspension cells or plants, three potential determinants of bacteria pathogenicity have been

found. These were, an extracellular polysaccharide (xanthan), a pectate lyase, and a toxin (Flood et al. 1995).

To date the interaction between elicitors of defence responses and cassava cells has not been exploited as a tool to explore the potential responses of the plant to pathogens or components of pathogens. This is the primary focus of this thesis. However, prior to carrying out such work the experimental conditions had to be optimised, in particular the establishment and characterisation of the plant suspension cells and the selection of the elicitor(s). Therefore, this chapter focuses on the establishment of the cassava suspension cells and the evaluation of different elicitors in order to select the appropriate elicitor(s) for detailed experimental work.

## 2 RESULTS

### 2.1 ESTABLISHMENT OF CASSAVA CELL SUSPENSION CULTURES

Cassava suspension cultures were initially obtained with Murashige and Skoog salt-medium composition (MS), supplemented with, 2% sucrose and 4 mg/l of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) following previous work (Deshappriya 1992). However, despite this hormone level proving successful for establishing cassava callus and suspension cells, preliminary experiments showed that, many defence responses were lacking or of low magnitude. Similar effects have been found in other plant cell culture / elicitor systems, which were resolved by reducing the auxin concentration (Dixon & Fuller 1976). Therefore, an experiment was designed in order to select a lower level of auxin that retained the capacity to induce callus formation and subsequently suspension cells. The auxin levels were chosen based on experiences with

other plant systems (Bohlmann & Eilert 1994; Dixon & Fuller 1976; Hohlfeld et al. 1995).

Each treatment consisted of five Petri dishes containing MS solid medium and a range of concentrations of 2,4-dichlorophenoxyacetic acid (1, 1.5, 2, 2.5, 3, 3.5, and 4 mg/l), each with five petioles (4-5 mm long) chosen at random. Once callus had developed, 0.01 g of fresh callus for each treatment replicate was weighed under sterile conditions and transferred to new Petri dishes containing the same medium for 2 weeks **(Figure 3.1)**. The effects of 2,4-D concentration on fresh and dry weight of callus were analysed using one way ANOVA and Duncan's mean test comparison **(Figures 3.2, 3.3).** For detail of statistics see **Appendix 1**.

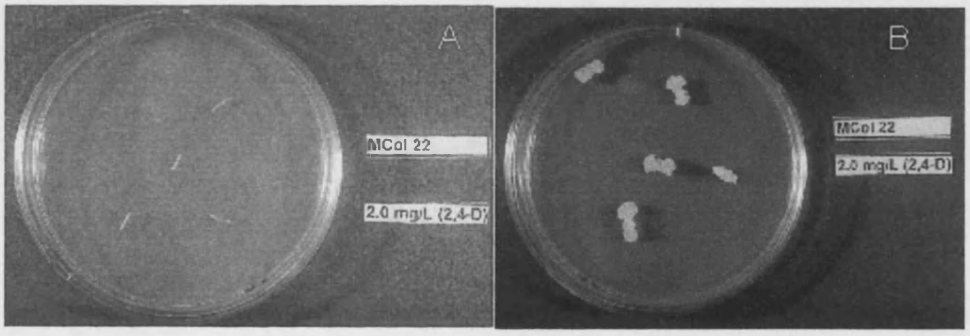



**Figure 3.1 Petioles and callus material of MCol 22 grown on 2.0 mg/l of 2-4,D.**

**A** – Photos taken just after transferring petioles to medium.

**B** – Photos of petioles taken two weeks after initiation showing callus material.

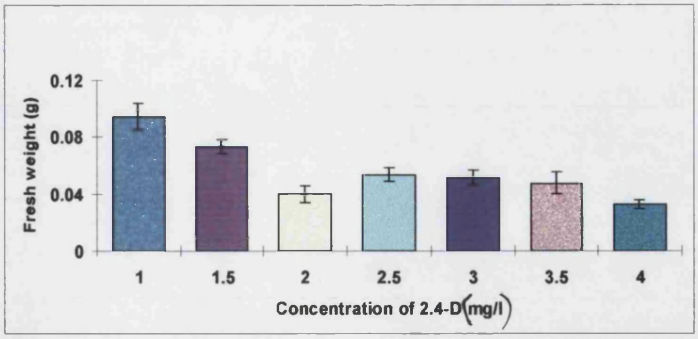



**Figure 3.2 Effects of different concentrations of 2,4-D on growth (fresh weight) of cassava callus per petiole.**

Error bars show standard error of means from five replicate plates each of five petioles.

The analysis of variance showed significant differences in fresh weight ($F_{6,168}$ = 13.51, < 0.001) between the treatments. Duncan's test showed that callus growing at low concentrations (1 and 1.5 mg/l) of 2,4-D produced significantly more biomass than those growing at higher concentrations. The means between 1 and 1.5 mg/l were not significantly different ($p \geq 0.05$), neither were those between 2, 2.5, 3, 3.5, and 4 mg/l auxin levels ($p \geq 0.05$).

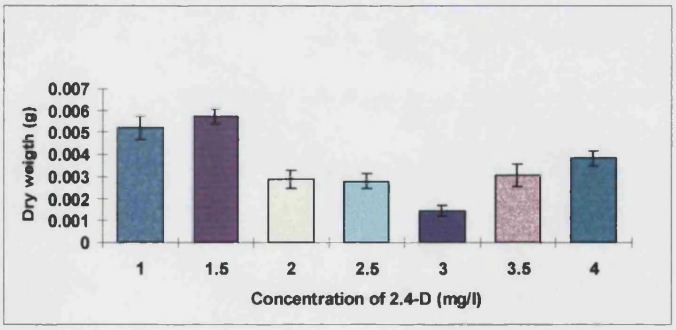



**Figure 3.3 Effects of different concentrations of 2,4-D on growth (dry weight) of cassava callus**

Error bars show standard error of means from five replicate plates each of five petioles.

The dry weights were measured, as it was necessary to test if the increment in biomass reflected an increase due to cell division, rather than the water content of the cells. Each callus was dried at 80 °C until constant weight. The analysis of variance showed that while there were no significant differences between the treatments with 1 and 1.5 mg/l 2,4-D, there were significant differences between these two and all the other treatments ($F_{6,168} = 13.51$, $p < 0.001$). Duncan's test showed that the means between 1 and 1.5 mg/ml (2,4-D) were not significantly different ($p \geq 0.05$), and that the means between the rest of the treatments were also not significantly different ($p \geq 0.05$). Therefore, the difference observed between the two treatments with the lowest concentrations of 2,4-D and the rest of the treatments was indeed due to increased biomass rather than increased water content.

While these results imply that 1 or 1.5 mg/l 2,4-D gave the greatest increase in biomass, 2.0 mg/l was chosen for all further experiments because several authors have shown that the lowest concentrations of auxin usually require the medium to be supplemented with other hormones (Dixon 1985; Krikorian 1991).

## 2.2 CHARACTERISTICS OF CASSAVA SUSPENSION CULTURES

Cassava suspension cultures were obtained by the transfer of friable callus pieces **(Figure 3.4)** to shaken liquid sterile medium of the same composition as that used for callus growth, but without agar. Cell suspension cultures require regular subculture and at more frequent intervals than the callus cultures from which they were derived (Dixon 1985).

Cell suspension cultures may be monitored by one or more different measurements, such as packed cell volume (PCV), cell number, wet and dry weights, protein and/or DNA content, conductivity of the medium, pH of the medium, and cell viability (Dixon 1985; Mroginski & Roca 1991).

The viability of the cells (**Figure 3.5),** the measurement of the packed cell volume (PCV) **(Figure 3.6)** and change of pH **(Figure 3.7)** were chosen to monitor growth of the cassava suspension cultures.

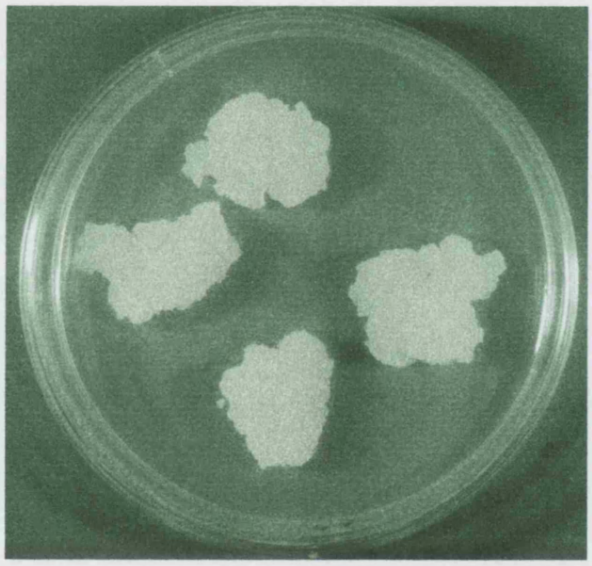


**Figure 3.4 Callus material from MCol 22 cassava cultivar.**

Photo taken one month after initiation of culture on 2.0 mg/l 2,4-D.

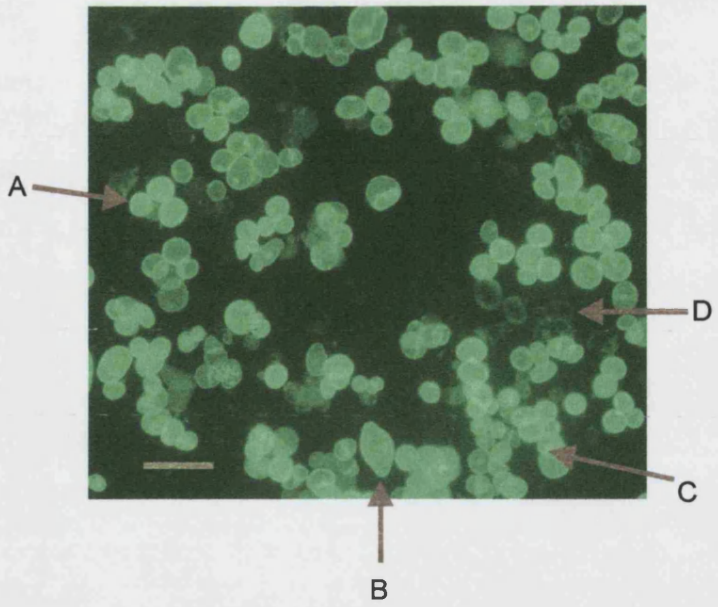



**Figure 3.5 Cassava cell suspension culture stained with FDA.**

Photo taken on the 5th day after sub-culture on 2.0 mg/l 2,4-D.

**A** – living cells fluorescing green; **B**- large cells which were observed during the time course of growth; **C** – cell aggregates; **D** – dead cells. Bar represents 50 μm.

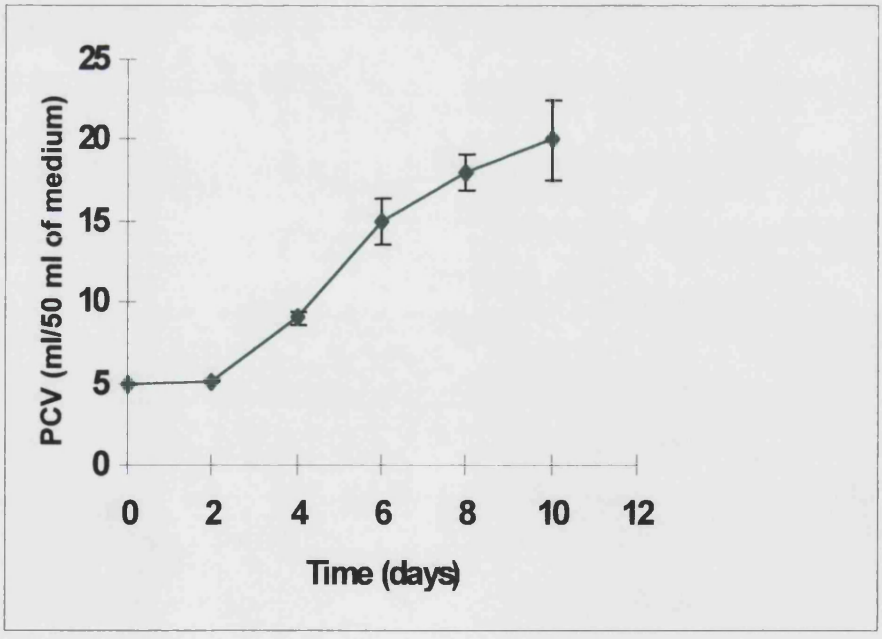



**Figure 3.6 Growth of cassava suspension cultures with time.**

Error bars show standard error of means with three replicates.

The increase in PCV over time was monitored in 50 ml of medium. Every 6-7 days 4.5-5 ml of cells were sub-cultured into 50 ml of fresh medium. The cells followed the standard pattern of growth found in other plant cell cultures, an initial lag phase between 0-2 days after sub-culture, exponential phase between days 2-6, early stationary phase between days 8-10, and finally late stationary phase from day 12. In general, cells from the period extending from late lag phase until mid-exponential phase provide the best starting material for experiments with elicitors in cell suspension cultures from a range of plant species (Bolwell et al. 1985a; Dixon 1985). Therefore, 5 days after sub-culture was chosen as an appropriate time point for elicitation of cassava cells for the work in this thesis.

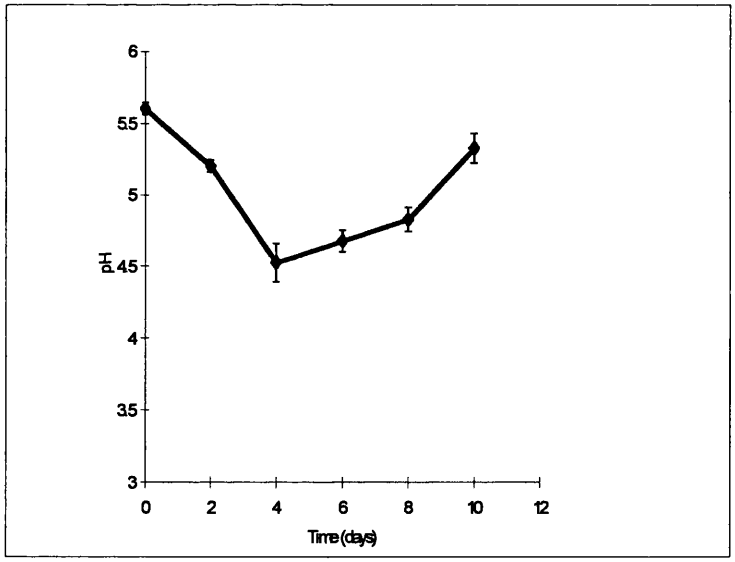



**Figure 3.7 Change in pH of cassava suspension culture versus time.**

Error bars show standard error of means with three replicates.

Prior to autoclaving, media had a pH 5.8; after autoclaving this dropped marginally to 5.6-5.7. During cell growth the pH of the culture decreased and

then increased with time **(Figure 3.7)**. The changes in pH became more consistent once the suspension cells had passed through at least 10-12 subcultures, after which the suspension cultures presented a more stable pattern of growth and change in pH.

In an attempt to improve growth of the cells by keeping the pH of the medium at 5.8 after autoclaving, the pH was raised to 6.0-6.2 before autoclaving. Cells grown in this adjusted medium showed considerable changes in shape, lacked the capacity for cell division and only remained viable for two days **(Figure 3.8)**. Therefore, the pH of the medium was adjusted to no more than 5.8 before autoclaving.

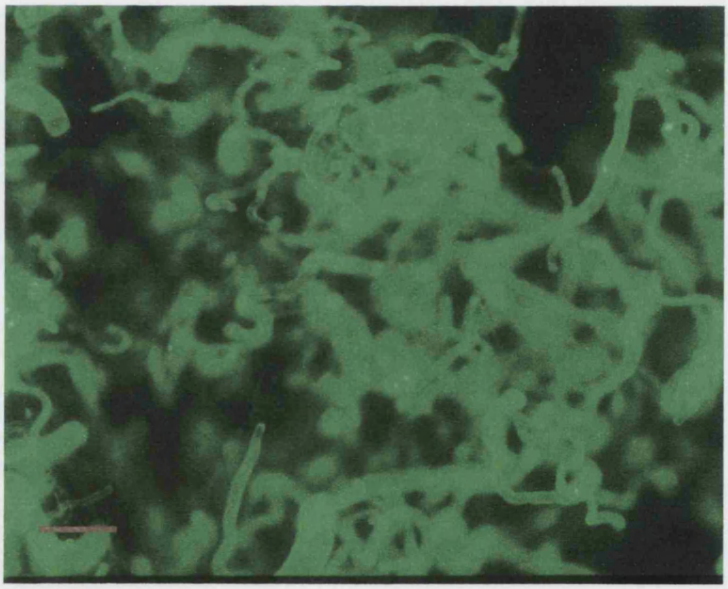


**Figure 3.8 Cassava suspension cells grown in medium with high pH.**

Bar represents 50 μm.

## 2.3 EFFECT OF ELICITORS ON EXTRA-CELLLAR pH OF CASSAVA SUSPENSION CELLS

In cultured cells, the primary responses evoked by the contact with elicitors can be grouped into two. An oxidative burst and a perturbation of the cellular ionic balance i.e. efflux of $K^+$ and $Cl^-$, influx of $Ca^{2+}$, external alkalinisation, and cellular acidification (Mathieu et al. 1994; Roos et al. 1998; Wojtaszek 1997). Elicitors effective in inducing defence-responses usually generate a pH change in cell suspension media (Baier et al. 1999; Tenhaken & Rübel 1998). Therefore, pH change of the medium induced by the addition of elicitors to cassava cell suspension cultures and the oxidative burst were used as a tool to assist in the choice of an appropriate elicitor for use in future experiments. The range of elicitors are used are listed in Table 3.1. Full results with each elicitor are presented in further figures **(Figures 3.9 – 3.15)**.

| Elicitor | Concentrations tested |
|---|---|
| Arachidonic acid | 0.01, 0.04, 1, 1.5 and 3 mM |
| *C. lindemuthianum* [A] | 18; 36; 42 μg/ml of glucose equivalents |
| Glutathione | $10^{-5}$ M; 0.5 mM; 0.1 mM; 1.5 mM |
| Jasmonic acid | $10^{-3}$ M; $10^{-4}$ M; $10^{-5}$ M; $10^{-6}$ M |
| Salicylic acid | 0.1 mM; 0.5 mM; 1 mM; 2 mM |
| *Xam* [B] | $10^{6}$; $10^{7}$; $10^{8}$ cfu |
| Xanthan | 0.02; 0.03; 0.05; 0.1 mg/ml |
| Yeast extract [C] | 50; 100; 200 μg/ml of glucose equivalents |

**Table 3.1 Elicitors evaluated for their effect on the change of pH in cassava cell suspension cultures.**

**A**: cell wall glucan elicitor from *C. lindemuthianum*; **B**: autoclaved *X. axonopodis* pv. *manihotis* (isolate 2967); **C**: cell wall glucan elicitor from baker's yeast preparation.

Different pHs in the cultures at the time zero are apparent in some of the graphs **(Figures 3.9-3.15)**. This is due to these experiments being some of the first group of experiments performed with cassava suspension cells challenged with elicitors, before the necessity of passing through several (10 – 12) subcultures of the cells in order to generate a more consistent behaviour had been recognised.

Arachidonic acid as elicitor did not induce any change in pH's at any of the concentrations tested. Cell wall glucan elicitor from *C. lindemuthianum* induced a rapid and brief alkalinisation of the medium within five minutes of elicitation at all the concentrations tested, followed by a progressive acidification; the optimal concentration was 42 μg/ml **(Figure 3.9)**. Results with glutathione **(Figure 3.10)** were unfortunately erratic; the initial pH of the cultures varied between experiments and the responses with different elicitor concentrations did not reveal any consistent pattern. Therefore, it was not possible to draw any useful conclusions from these particular results. Jasmonic acid induced pH changes at all concentrations and the recognition of the elicitor seemed to be within 5 to 10 minutes after elicitation; the optimal concentration was $10^{-5}$ M **(Figure 3.11)**. Salicylic acid elicitor showed an increase in pH with three of the concentrations tested, but this increase in pH was only observed between five to six hours after elicitation **(Figure 3.12)**. Xanthan elicitor showed erratic responses with all the concentrations tested; the highest alkalinisation of the medium was observed with 0.02 mg/ml after one hour **(Figure 3.13)**. Autoclaved *Xam* as elicitor induced an alkalinisation of the medium with all concentrations tested after one hour on initiation, with an optimal concentration of $10^{8}$ cfu/ml **(Figure 3.14)**. Glucan cell-wall from baker's yeast induced an

increase in pH with all the concentrations tested within the first three hours of elicitation; this change was most pronounced at the lowest (50 μg/ml) concentration **(Figure 3.15)**.

The importance of having the cells at the same initial physiological state, so as to ensure the same initial pH, was noted. This can be achieved through passing the cells through several (10 - 12) subcultures before using them for experimentation. However, despite this, all the elicitors tested, with the exception of arachidonic acid, induced an alkalinisation of the medium. In most cases, notably with *C. lindemuthianum* and yeast, this was transient and dose dependant. No elicitor induced a second, later, alkalinisation of the medium.

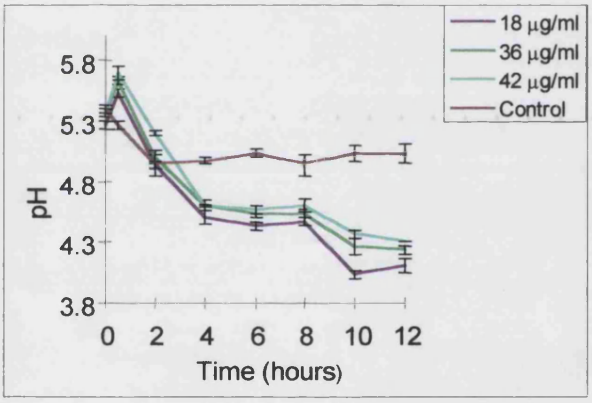



**Figure 3.9 Effects of *Colletotrichum lindemuthianum* glucan cell-wall on the change of pH in cassava cell suspension culture over a time course.**

Error bars show standard error of means with three replicates. Concentrations used were based on those from (Bolwell et al. 1985b).

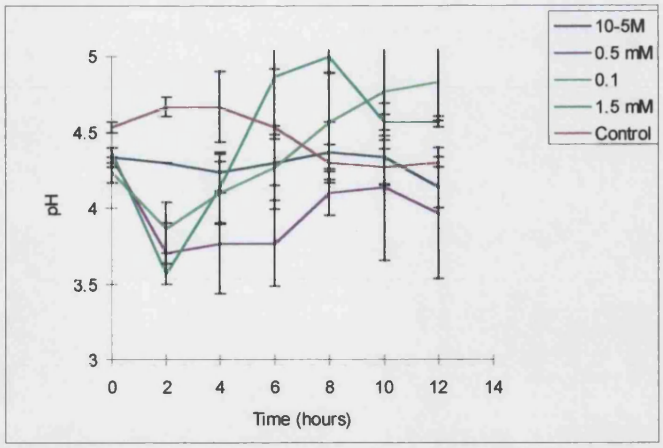



**Figure 3.10 Effects of glutathione on the change of pH in cassava cell suspension culture over a time course.**

Error bars show standard error of means with three replicates. Concentrations used were based on those from (Guo & Ohta 1993; Guo et al. 1993; Nakagawara et al. 1993; Wingate et al. 1988).

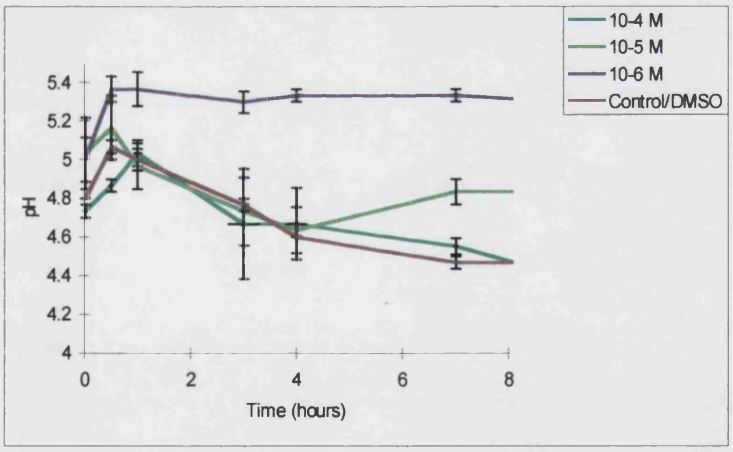



**Figure 3.11 Effects of jasmonic acid on the change of pH in cassava cell suspension culture over a time course.**

Error bars show standard error of means with three replicates. Concentrations used were based on those from (Gundlach et al. 1992).

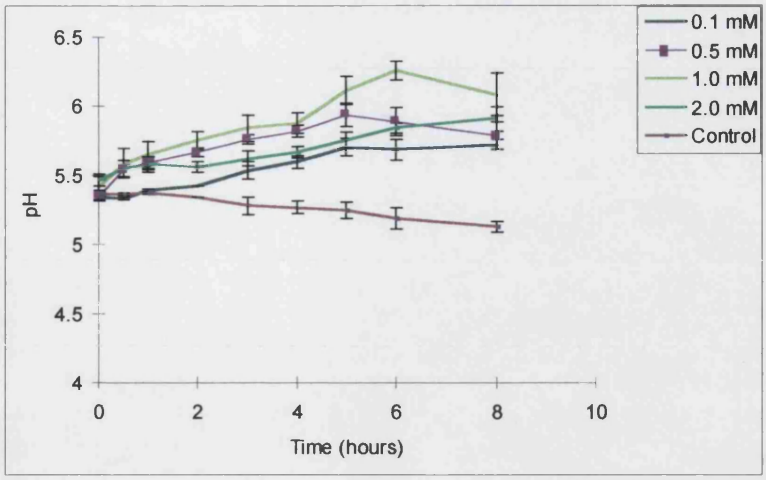



**Figure 3.12 Effects of salicylic acid on the change of pH in cassava cell suspension culture over a time course.**

Error bars show standard error of means with three replicates. Concentrations used were around on those from (Umetani et al. 1990).

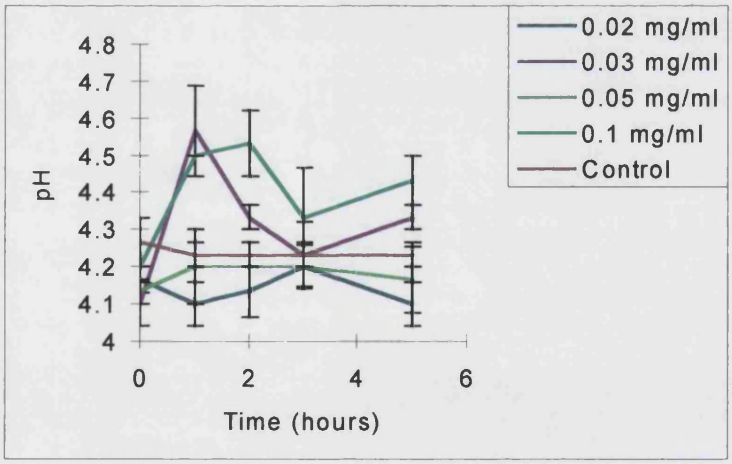



**Figure 3.13**

**Effects of xanthan on the change of pH in cassava cell suspension culture over a time course.**

Error bars shows standard error of means with three replicates.

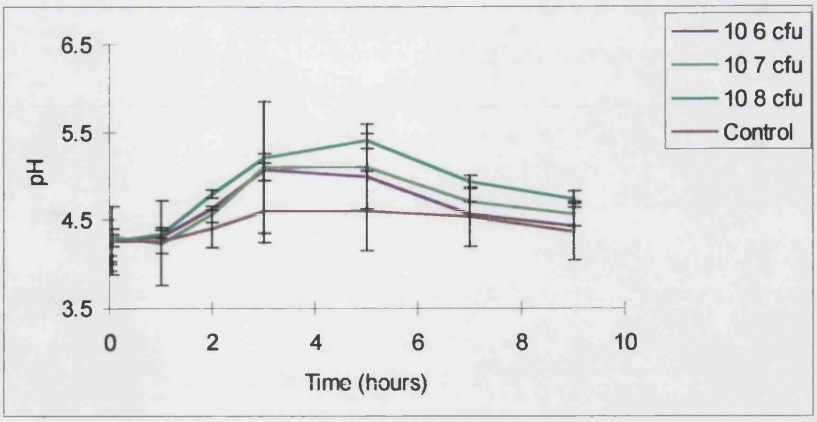



**Figure 3.14 Effects of autoclaved *X. axonopodis* pv. *manihotis* on the change of pH in cassava cell suspension culture over a time course.**

Error bars shows standard error of means with three replicates. Autoclaved bacteria were used as elicitor isolated 2967. Elicitor concentrations in cfu/ml.

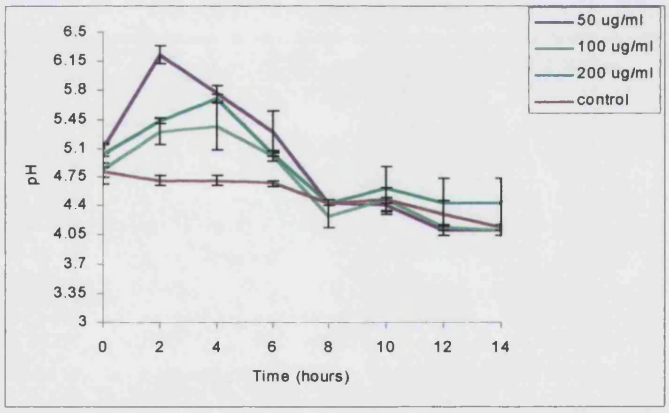



**Figure 3.15 Effects of baker's yeast glucan cell-wall elicitor on the change of pH in cassava suspension cells over a time course.**

Error bars show standard error of means with three replicates. Concentrations used were based on those from (Bohlmann et al. 1995; Guo & Ohta 1993).

## 2.4 PRODUCTION OF $H_2O_2$ IN CASSAVA SUSPENSION CELLS CHALLENGED WITH ELICITORS

Detection of an oxidative burst was used with a range of elicitors as another tool for the better selection of effective elicitor(s). Three more elicitors were tested, in addition to those used previously, oligalacturonic acid (OGA), lypopolysaccharide from *X. axonopodis* pv. *manihotis* (isolate St03) (LPSx) and lypopolysaccharide from *Pseudomonas* pv. *syringae* (LPSp). The results obtained are summarised in the table but further experiments on the oxidative burst will be described in the following chapter (**Table 3.2**). Six of the elicitors tested induced an oxidative burst; however, arachidonic acid, jasmonic acid and salicylic acid did not.

| Elicitor | Concentration tested | $H_2O_2$ production |
|---|---|---|
| Arachidonic acid | 3.0 M | no |
| *C. lindemuthianum* [A] | 42 μg/ml | yes |
| Glutathione | 1.5 mM | yes |
| Jasmonic acid | $10^{-5}$ M | no |
| LPS from *Ps.* [B] | 200 μg/ml | no |
| LPS from *Xam* [C] | 200 μg/ml | yes |
| Oligalacturonic acid (OGA) | 7.5 μg/ml of uronic acid equivalents | yes |
| Salicylic acid | 1 mM | no |
| Yeast extract [D] | 50, 100 and 200 μg/ml of glucose equivalents | yes |

**Table 3.2 Production of $H_2O_2$ in cassava suspension cells-challenged with elicitors.**

**A**: Cell wall glucan from *C. lindemuthianum*; **B**: Lipopolysaccharide from *Pseudomonas* spp; **C**: Lipopolysaccharide from *X. axonopodis* pv. *manihotis* ; **D**: Cell wall glucan from yeast.

# 3 DISCUSSION

Cassava petioles produced callus on media containing a range of concentrations of the auxin (2,4-D). Greatest callus biomass was produced at the lowest concentrations of 1 and 1.5 mg/l of (2,4-D). However, for long term cell suspension cultures of other plants such low concentration of 2,4-D often have to be supplemented with other hormones (Dixon 1985; Krikorian 1991). While all concentrations of 2,4-D tested resulted in callus growth, preliminary experiments with cassava suspension cells suggested that the higher concentrations led to inconsistent defence responses. Other work has shown that the concentration and balance of growth regulators, and the medium composition, affect different enzymes (e.g. PAL and polyphenol oxidase) and the expression of secondary metabolism in cultured plant cells (Eilert 1987; Yeoman & Yeoman 1996). High levels of synthetic auxin strongly affected cell growth, inhibited PAL activation and decreased production of the phytoalexin phaseollin in bean (*Phaseolus vulgaris* L.) suspension cells (Dixon & Fuller 1976). Furthermore, bean suspension cultures grown in the presence of 2,4-D accumulated lower levels of phaseollin than those grown with naphthaleneacetic acid (NAA) (Dixon & Fuller 1976). 2,4-D in the medium of *Morinda citrifolia* suspension cells had an effect on growth, resulting in high cell division rate and metabolic activity, but not on the production of anthraquinones (van der Plas et al. 1995). On the other hand, phytoalexin production (6-methoxymellein) in *Daucus carota* cultures was promoted by the auxin 2,4-D compared to that with indoleacetic acid (IAA) (Kurosaki & Nishi 1983). Carrot suspension cells without auxin in the medium did not accumulate detectable amounts of phytoalexin after elicitation (Kurosaki et al. 1985). Under auxin

starvation, bean suspension cells-challenged with 10 μg/ml of oligogalacturonides (DPs 9-18) did not secrete a 32kD protein (which regulates growth and development), which is accumulated in the culture medium in the presence of auxin (Bellicampi et al. 1995)

Not only the balance between hormones can affect the accumulation of secondary metabolites and enzyme activities in plant suspension cells, the quantity of the carbon source in the medium can also play an important role. Carrot suspension cells synthesised anthocyanin and showed PAL gene activity (ANT-PAL) in a nutrient medium containing zeatin instead of 2,4-D and a high level of sucrose (4%) (Ozeki & Takeda 1994). Generally, raising the initial level of sucrose has led to an increase in secondary metabolites or stimulation of the activity of some enzymes in plant suspension cells (Yeoman & Yeoman 1996); these authors found that several other conditions affected the growth and behaviour of plant suspension cells, including nutrients, light, temperature and aeration.

The results presented in this chapter, together with an evaluation of the literature, suggest that a concentration of 2 mg/l of 2,4-D is a good compromise for the growth and maintenance of cassava suspension cells and for the analysis of defence responses induced by elicitors. Cassava suspension cells can be obtained and subcultured with 2 mg/l of the auxin 2,4-D, under continuous subculture every 6-8 days. However, for each cell culture line there is a minimum inoculum size below which the culture will not grow (Dixon 1985). 4.5-5 ml of cells in 50 ml of medium every 6-8 days was adopted as a convenient quantity for regular subcultures. The minimum cell density is called

a 'feeder effect' because the deficiencies of cells can often be made up by the presence of other cells growing nearby (George & Sherrington 1984).

Cassava suspension cells included groups of cell aggregates during their growth cycle; this phenomenon has been reported in other plant suspension cultures and the proportion and size of cell aggregates varies according to the plant variety and the medium in which the culture is grown (Mroginski & Roca 1991). Additionally, plant cultures propagated under continuous culture in fermenters consisted of very fine suspensions of single cells and of small cell aggregates. This phenomenon has been attributed to the good aeration of the cultures, which contrasts with that of suspension cells grown in Erlenmeyer flasks, which have an increased tendency to form aggregates. Furthermore, fermenters permit higher production of secondary metabolites and enzymes compared to batch culture (Hahlbrock et al. 1974). Additionally, not only aeration and the balance between hormones have been implicated in generating cell aggregates in suspension cells, but also the exhaustion of nutrients in the medium. For instance in carrot suspension cells, if $Mg^{2+}$ was a limiting nutrient there was an increase in the size and number of cell aggregates (Dougall et al. 1983).

Plant suspension cultures can survive in a wide range of pHs depending on the plant species and culture conditions. For instance, pH 6.25 has been reported to be the optimum for carrot suspension cells (Steward & Bleichert 1972). Usually, the pH reported in the medium of different plant suspension cells is between 4.0-5.5 (Krikorian 1991). However, cassava suspension cells grown above pH 5.8 had different cell morphology, a dramatic decrease in growth and rapid cell death compared to cells grown at lower pH values. The

pH of the culture medium can influence the uptake of nutrients and precursors, and change the permeability of plant membranes, leading to the release of products from the vacuole into the culture medium (Yeoman & Yeoman 1996). Substances known to be released into the medium by cultures include alkaloids, amino acids, enzymes, growth substances and vitamins (George & Sherrington 1984). The pH affects ammonium uptake rates in carrot cell cultures and aggregate size distribution (Steiner & Dougall 1995). During the course of growth, plant cells in culture change their pH; this fluctuation in pH is thought to arise from the metabolic activity of the cells, but dramatic pH changes of more than 2.0 units have been observed in cell cultures (Hahlbrock et al. 1974) . A stable pH occurs when the cells reach stationary phase, which is due to the exhaustion of the nutrients in the medium (Steiner & Dougall 1995).

The measurement of the extracellular pH has been used to monitor events related to defence response mechanisms in plants, such as the hypersensitive response in tobacco suspension cells to the phytopathogenic bacterium, *P. syringae* pv. *syringae*, which induces an increase in extracellular pH at the onset of the hypersensitive reaction, (Dale et al. 1989). Alkalinisation of the growth medium has been described as an early response of suspension-cultured cells challenged with bacterial pathogens (Felix et al. 1993; García-Pérez et al. 1998; Mathieu et al. 1996; Wei et al. 1992). It has been suggested that proton-pumping ATPases may be involved in the alkalinisation of the culture medium in elicited cell culture systems (Hagendoorn et al. 1991; Sacks et al. 1993; Wei et al. 1992). Furthermore, this proton import may be associated with $K^+$ efflux and $Ca^{2+}$ influx, membrane depolarisation and cytosolic acidification (Sacks et al. 1993; Mathieu et al. 1994). The intensity

and the kinetics of the alkalinisation of the medium can differ according to the elicitor used in a given plant suspension culture. For instance, alkalinisation of the extracellular medium induced by oligogalacturonide elicitors reached a maximum increase in pH after 50 min, this contrasted with 150-200 min with *P megasperma* elicitor in the same tobacco suspension cells. On the other hand, the *P. megasperma* elicitor induced a long-lasting extracellular alkalinisation (Mathieu et al. 1994). There is also a correlation between the timing of the rise of the external pH and the generation of hydrogen peroxide in alfalfa, bean, tobacco and soybean suspension cells (Baier et al. 1999; Bolwell 1995; Pugin & Guern 1996; Tenhaken & Rübel 1998).

A variation of culture pH was observed during the initial subcultures of the cassava cells, which interfered with some of the initial experiments. This pH stabilised after several (10 – 12) subcultures. No reference to this phenomenon was found in the literature. For all the subsequent experiments reported in this thesis cassava cell suspensions of a at least 10 subcultures were used.

There is a probable link between pH changes and protein phosphorylation, based on evidence that K-252a and staurosporine (both inhibitors of protein kinases) blocked both events in tomato suspension cells (Felix et al. 1993). The possible role of the ionic and electrical changes, such as increase in concentration of cytosolic $Ca^{2+}$, alkalinisation in the extracellular pH, acidification of the intracellular pH, increase in the intracellular phosphate concentration could be secondary signals in signal transduction pathways leading ultimately to changes in gene expression (Mathieu et al. 1994). Calcium ions may also be involved in the transduction of elicitor signals. For instance, if the extracellular $Ca^{2+}$ was removed by the chelator ethylene glycol

bis(b-aminoethyl ether) N,N'-tetraacetic acid in onion suspension cells challenged with *Botrytis cinerea* elicitor, the onion suspension cells failed to induce the phytoalexins 5-hexyl-cyclopenta-1,3-dione and 5-octyl-cyclopenta-1,3-dione (Dmitriev et al. 1996). Similar results were obtained with tobacco suspension cells treated with cryptogein (a proteinaceous elicitor from *Phythophtora cryptogea*) and the same inhibitor; these cells failed to produce phytoalexin and an alkalinisation of the medium (Tavernier et al. 1995). Supporting evidence for the role of calcium ions come from work with the $Ca^{2+}$ ionophore A23187, which has been shown to stimulate phytoalexin accumulation in soybean (Stab & Ebel 1987).

All elicitors tested on cassava suspension cells generated an alkalinisation of the extracellular medium, except for arachidonic acid. On the other hand, no pH changes were detected in soybean suspension cells treated with either polygalacturonic acid or with an elicitor preparation from *Verticilium dahliae* . Yeast elicitor produced an alkalinisation of the cassava extracellular medium greater than that reported in tomato cells challenged with yeast elicitor, in which 0.6 pH units was reached after 0.2-8 minutes after elicitation (Felix et al. 1993) compared to the 1.3 pH units observed with cassava suspension cells here. These authors concluded that the β-glucan was not responsible for this change in pH in tomato cells, but rather chitin contamination in the preparation of their elicitor. It should be pointed out that the yeast glucan cell-wall elicitor used here with cassava was prepared by a different method (Schumacher et al. 1987) than that used with tomato (Felix et al. 1993). Alfalfa and tobacco suspension cultures also responded to yeast elicitor by an alkalinisation of the medium in a dosage-dependent manner (Baier et al. 1999). The results

presented in this chapter with yeast are in disagreement with their observations that the pH started to increase about 5 minutes after addition of the elicitor and reached a peak value in 15-20 minutes; with cassava a peak was observed by two hours. However, their peak value of almost 0.8 pH units is in agreement of observations presented here with cassava suspension cells (Baier et al. 1999). It should be pointed out that the concentrations used here with cassava cells (5-200 μg/ml of glucose equivalents) are considerably lower than the 500 μg/ml used with tobacco. Other elicitors can cause pH changes after time periods considerably greater than those observed in cassava cells. For example, *Capsicum annuum* suspension cultures treated with mycelium from *Phytophthora capsici* showed an increase in pH after 24 hours in more than 1.5 pH units (García-Pérez et al. 1998).

The initial selection of elicitors was based on literature research. It has been suggested that chemically defined elicitors generate less variation in the reproducibility of the results than complex elicitor preparations (Eilert 1987). The cell walls of *Phytophthora* species contain arachidonic acid (Altamura et al. 1994), and *Phytophthora drechsleri* produce soft rot in cassava roots (Lozano, Bellotti, Reyes, Howeler, Leihner, & Doll 1981). However, as relatively high concentrations of arachidonic acid were needed for elicitation (e.g. 3.3 mM to induce terpenoid accumulation in potato), it is thought to act synergistically with other *Phytophthora* glucan elicitors (Dixon 1986). However, this elicitor was discarded because it failed to change in pH or oxidative burst in cassava cells, for similar reasons jasmonic acid and salicylic acid were discarded. Autoclaved *Xam*, although effective, was discarded as a putative elicitor because of its

undefined, possibly, irreproducible composition. In all elicitors tested, the earliest and maximum levels of pH change occurred with the cell wall glucan from yeast and the cell wall glucan from *C. lindemuthianum*. The fact that a cell wall glucan from *C. lindemuthianum* and yeast preparation elicitors triggered an alkalinisation in cassava suspension cells at all concentrations tested, suggests that cassava recognised these glucans in a non-specific manner. However, it may be noted that cassava suffers from anthracnose, a disease caused by *C. gloeosporioides* (Fokunang et al. 1999). However, proportionately small quantities of *C. lindemuthianum* elicitor are obtained from large amounts of fungal mycelium (C. Gerrish, personal communication). Therefore, a practical approach was to select an elicitor that can be produced readily in large quantities that can be kept stable for a long period of time. For the above reasons cell wall glucan from yeast was selected. Furthermore, the capacity of the cell wall glucan from yeast to act as elicitor has been observed in suspension cultures from a wide range of plant species (Chen & Chen 2000; Dey et al. 1997; Brownleader et al. 1997; Chen & Chen 2000; Song et al. 1995; Yamamoto et al. 1995; Wojtaszek et al. 1997).

Cassava suspension cells with 2.0 mg/l of 2,4-D were established and the conditions for the growth were standardised. The alkalinisation response and hydrogen peroxide production monitored. Different elicitors were tested and a transient alkalinisation of the extracellular medium was observed in all of them except, arachidonic acid. An early alkalinisation of the extracellular medium was only observed with two elicitors, cell wall glucan from yeast and cell wall glucan from *C. lindemuthianum*. Recognition of these cell wall glucans by cassava suspension cells appears to occur in a non-specific manner. An

oxidative burst was observed with all elicitors tested except for salicylic acid, arachidonic acid and jasmonic acid. This production of hydrogen peroxide was observed after 5 min of elicitation and peaked at 25-30 minutes after elicitation. The change in pH and oxidative burst in cassava suspension cells with elicitors appears to be transient and dose-dependent events.

# CHAPTER 4

# MODULATION OF REACTIVE OXYGEN SPECIES IN ELICITOR-CHALLENGED CELLS

## 1 INTRODUCTION

Oxygen and the reactive oxygen species (ROS) derived from it are potentially damaging to all life forms including plants. Therefore, organisms counter such oxidative stress by restricting their boundaries in order to control access of oxygen to their tissues and by synthesizing enzymes and other molecules that can modulate or detoxify ROS. These detoxifying mechanisms include enzymes such as superoxide dismutase, catalase and peroxidase, and antioxidant compounds such as glutathione, phenolic compounds and ascorbate (Larson 1995; Scandalios 1990). However, while ROS are potentially damaging to the plant and require detoxification, they can also be beneficial; they are a double-edged sword (Bowler et al. 1994). ROS are involved in the cross-linking of cell wall proteins such as hydroxyproline-rich glycoproteins (HRGPs), lignin biosynthesis, the induction of defence-related genes, the stimulation of phytoalexin biosynthesis and the promotion of the hypersensitive

response (HR) in incompatible plant-pathogen interactions (Low & Merida 1996).

In the previous chapter alkalinisation of the medium after elicitation was detected with some of the elicitors tested. This event and the activation of the oxidative burst are amongst the most rapid reflection of initial signaling events in plant-pathogen interactions that do not involve gene transcription. Oxidative burst events lead to the activation of defence responses, many of which require the transcriptional activation of specific genes (Mehdy 1994). Reactive oxygen species (ROS) are generated during the oxidative burst. ROS are produced at low levels during normal growth and development by plant cells in chloroplasts, mitochondria, and by enzymes in other cell compartments involved in reduction-oxidation processes (Wojtaszek 1997). Cells have the relevant protective mechanisms to maintain the lowest possible levels of ROS inside the cell. In some cases, however, especially under stress conditions, attack from pathogens, or elicitor induction, these protective mechanisms are overridden by the transient production of huge amounts of ROS, namely the oxidative burst. While the oxidative burst process has not been directly linked with the induction of phytoalexin accumulation for all plant systems, the involvement of ROS in lignification, oxidative cross-linking of cell wall proteins, as a second messengers, and in the regulation of the hypersensitive response has been demonstrated in a number of cases (Bradley et al. 1992; Davis et al. 1993; Devlin & Gustine 1992; Jabs et al. 1997; Wu et al. 1997).

The interaction between plant cell suspension cultures and elicitors mimics some components of plant-pathogen interactions, including the induction, action and detoxification of ROS. Therefore, aspects of the

interaction between cassava cells and yeast glucan cell wall that involve ROS were examined and are described in this chapter.

Production of hydrogen peroxide by cassava suspension cells challenged with some of the elicitors tested was observed. To study xanthine oxidase as a potential source of ROS an assay for activity was deployed and cloning of the gene(s) was attempted. Similarly, as one of the components in the production of the oxidative burst in some plants, peroxidase was examined by measurement of enzyme activity and of gene transcription by Northern analysis; also their capacity to oxidise scopoletin, one of the key phenolic compounds found in cassava cells, was studied. Peroxidase isoforms were visualized in IEF gels. Northern analysis was also performed with catalase an enzyme considered as a first line of antioxidant defence in plants. Expression of hydroxyrich-glycoprotein gene (HRGP) involved strengthening of the cell wall, was also examined. Finally, some antioxidant compounds from elicited cells were visualized on HPTLC plates.

## 2 RESULTS

### 2.1 OXIDATIVE BURST IN CASSAVA SUSPENSION CELLS CHALLENGED WITH DIFFERENT ELICITORS

In the previous chapter yeast was selected as an effective elicitor for detailed experimental work with cassava suspension cells based on its ability to increase the pH of the medium and other parameters. These data suggested that cell wall glucan from yeast had the ability to induce a wide range of defence-related responses in cassava cells including an oxidative burst. In order to confirm this, the oxidative burst induced by yeast was compared to that

induced by other elicitors **(Figure 4.1).** Of the nine elicitors tested only four, yeast cell wall glucan, *C. lindemuthianum* cell wall glucan, oligalacturonic acid and glutathione induced a substantial oxidative burst. These four elicitors triggered a rapid, transient oxidative burst, which started within 5 min and reached a maximum peak after 20-35 min and had decayed substantially by 60 min after elicitation. Although the figure shows the response over 65 min, it was followed for 4 h during which no secondary peak was detected. The hydrogen peroxide peak measured varied between 8.5 and 10.5 μM **(Figure 4.2)**. These data confirm yeast as an effective elicitor of a rapid oxidative burst in cassava suspension cells.

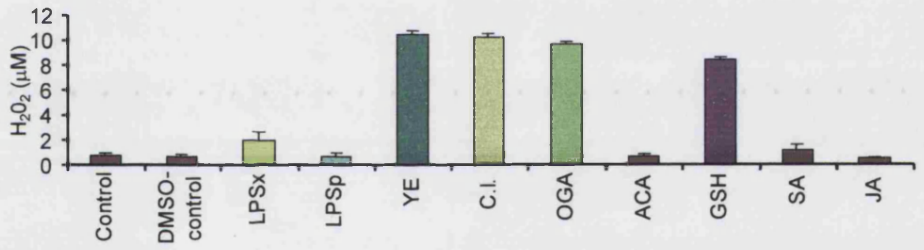



**Figure 4.1 Oxidative burst observed in cassava suspension cells challenged with several elicitors, 20 min after elicitation.**

**Control:** SDW-treated; **DMSO control:** Dimethyl sulfoxide treated; **LPSx**: Lipopolysaccharide from *Xanthomonas* strain St03 (200 μg/ml); **LPSp**: Lipopolysaccharide from *Pseudomonas* sp (200 μg/ml); **YE**: Cell wall glucan from yeast preparation (50 μg/ml of glucose equivalents); **C.I.**: Cell wall glucan from *C. lindemuthianum* (42 μg/ml of glucose equivalents); **OGA**: Oligalacturonic acid (7.5 μg/ml of uronic acids); **ACA**: Arachidonic acid (3 mM); **GSH**: Glutathione (1.5 mM); **SA**: Salicylic acid (1 mM); **JA**: Jasmonic acid ($10^{-5}$ M).

Error bars represent standard error of three independent replicas.

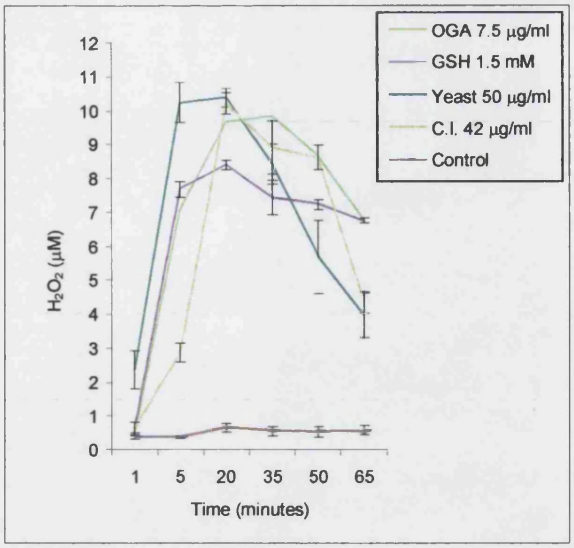



**Figure 4.2 Oxidative burst from cassava suspension cells challenged with different elicitors.**

**OGA**: Oligalacturonic acid (7.5 µg/ml of uronic acids); **GSH**: Glutathione (1.5 mM); **Yeast**: Cell wall glucan from yeast preparation (50 µg/ml of glucose equivalents); **C.I.**: Cell wall glucan from *C. lindemuthianum* (42 µg/ml of glucose equivalents); **Control**: SDW-treated control.

Error bars represent standard error of three independent replicas.

## 2.2 XANTHINE OXIDASE AND REACTIVE OXYGEN SPECIES

Xanthine oxidase (XO) is one of the potential sources for the formation of reactive oxygen species in plants (Slusarenko & Milosevic 1995). However, no clear evidence on the importance of this enzyme is available. Very little is known about the occurrence and properties of XO in plants. In animals, XO is an important component of oxygen-mediated tissue injury, because xanthine oxidase reduces oxygen to the cytotoxic intermediates superoxide and hydrogen peroxide (Beckman et al. 1989). In an attempt to shed light on the possible role of XO in the cassava oxidative burst, its activity in elicitor-

challenged cells was assayed. In addition a PCR-based strategy was adopted to try and clone the XO gene(s) from cassava.

### 2.2.1 Activity of Xanthine Oxidase in Cassava Suspension Cells

Xanthine oxidoreductase has been characterized in higher plants as a dehydrogenase. Xanthine dehydrogenase (E.C. 1.2.1.37), which normally reduces $NAD^+$, is converted to a xanthine oxidase (xanthine: oxidoreductase E.C. 1.2.3.2), which belongs to the molybdenum cofactor dependent hydroxylase class of enzymes. Xanthine oxidase (XO), aldehyde oxidase (AO; E.C. 1.2.3.1) and nitrate reductase (NR; E.C. 1.6.6.1) are the only three molybdenum dependent-enzymes that have been found in plants so far (Aukerman & Amasino 1996). Xanthine oxidoreductase catalyzes the first oxidative step in purine catabolism and is necessary for ureide biosynthesis in higher plants through *de novo* synthesis of purines from glutamine (Nguyen 1983; Triplett et al. 1982). The catalysis of xanthine by the enzyme (XO) can lead to the production of $H_2O_2$ (xanthine + $O_2^-$ + $H_2O \rightarrow$ urate + $H_2O_2$) (Owen & Johns 1999). In plants xanthine oxidase had been detected in peroxisomes and mitochondria (Pontier et al. 1999).

XO activity was measured in cassava cells over a time course following elicitation. In addition to cell wall glucan from yeast elicitor and from *C. lindemuthianum* elicitor, which both induced a substantial oxidative burst, LPS from *Xam* strain St03 and *Pseudomonas* sp., which did not, were also used as elicitors **(Figures 4.3-4.6)**. Activity appeared to be constitutive based on the similar activities in all treatments. However, results showed a considerable variation (see SE in **Figures 4.5, 4.6)**, which restricts interpretation. Although

activities were usually greater following elicitation of cells this did not reveal a temporal increase. Possibly one of the source of variation was the shaking-stress, which is characteristic of the suspension cell systems.

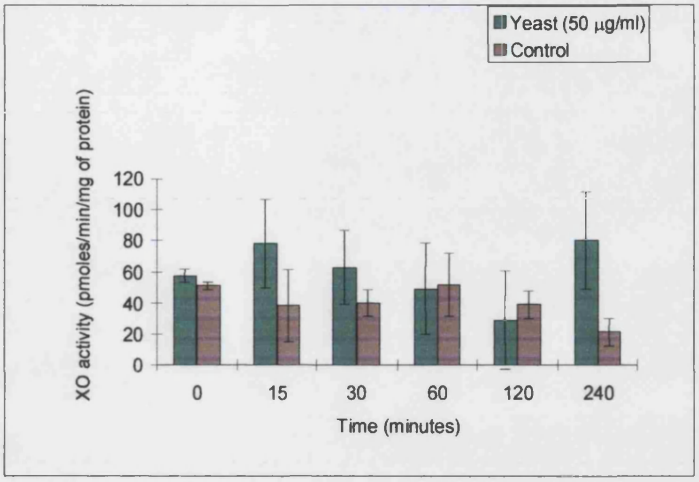



**Figure 4.3 Xanthine oxidase activity in cassava cells challenged with yeast elicitor.**

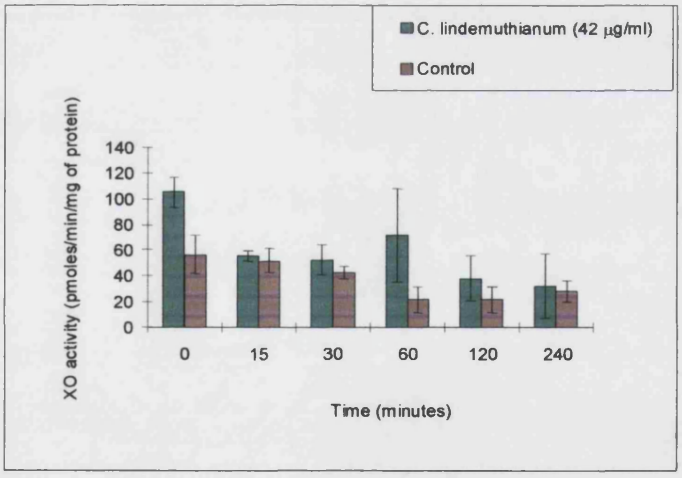



**Figure 4.4 Xanthine oxidase activity in cassava cells challenged with *C. lindemuthianum* elicitor.**

Error bars represent standard error of three independent experiments.

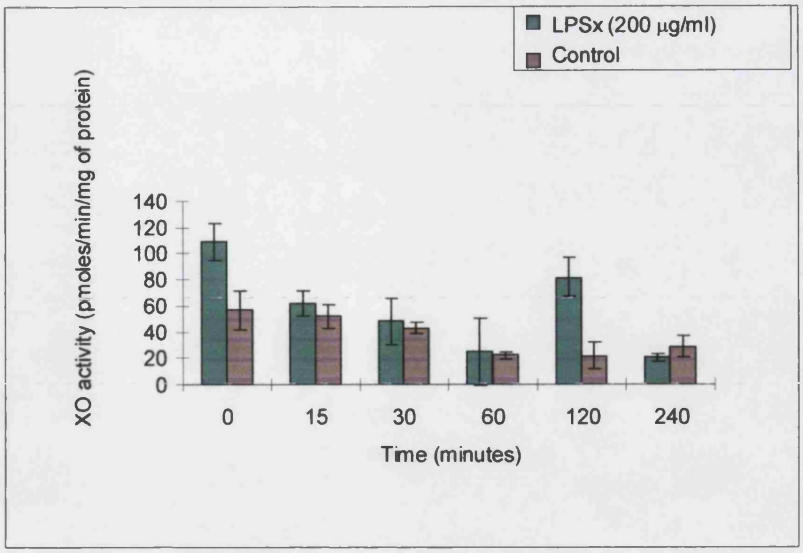



**Figure 4.5 Xanthine oxidase activity in cassava cells challenged with *Xanthomonas* LPS elicitor.**

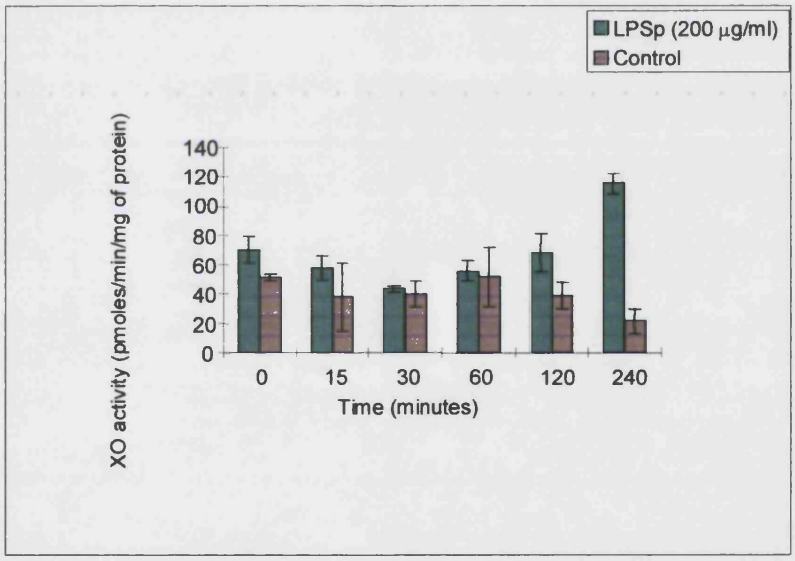



**Figure 4.6 Xanthine oxidase activity in cassava cells challenged with *Pseudomonas* LPS elicitor.**

Error bars represent standard error of three independent experiments.

2.2.2 Xanthine Oxidase Gene Cloning

Xanthine oxidase has not been cloned from plants, so no plant heterologous probe was available for screening cassava cDNA or genomic libraries. While XO has been cloned from animals and fungi there was sufficient divergence between these sequences to suggest that these clones were unlikely to prove successful as heterologous probes. Therefore, a PCR-based approach was adopted, relying on use of degenerate primers designed from conserved regions of available XO genes. XO DNA sequences from cow (*Bos taurus*), EMBL accession BTXZND; human (*Homo sapiens*), HSXDHA; chicken (*Gallus gallus*), GGXDHY; *Drosophila melanogaster*, DMXDH; and *Aspergillus nidulans*, ANHXA were used as a basis for designing primers as, due to their diversity, strongly conserved regions should be readily apparent in multiple sequence alignments.

Initially, the web-based GeneFisher (V. 1.1) software was used (http://bibiserv.techfak.uni-bielefeld.de/genefisher/). Gene Fisher requires pre-aligned data as input, such as is generated by Clustal W (Higgins DG et al. 1996), which it searches using a range of modifiable parameters, for the identification of best primer pairs. However, despite trying increasingly non-specific primer parameters, no primer pairs were predicted by the program. Therefore, a multiple alignment of the five XO sequences was generated by Clustal W, conserved regions identified and degenerate primers designed by eye **(Appendix 2)**. The guiding philosophy was that pairs of nested primers were most likely to lead to the amplification of parts of cassava XO genes; the outer pair of primers being used for primary and the inner pair for secondary amplification of the initial PCR products. PCR products would then be checked

by sequencing and BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1997) to determine whether or not they corresponded to XO sequences. A diagram of xanthine oxidase degenerate primers showing approximate positions is summarized below **(Figure 4.4)**. Approximate minimum band sizes with pairs of PCR primers could be predicted **(Table 4.1)**.

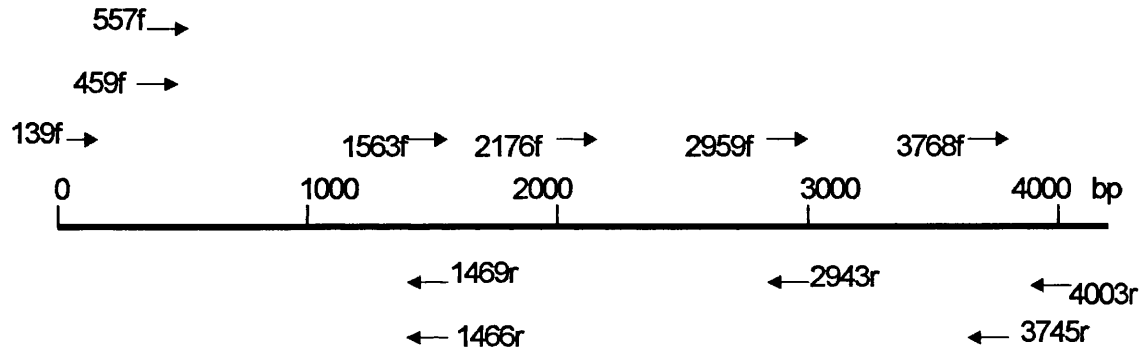



**Figure 4.7 Diagram of xanthine oxidase multiple alignment showing approximate positions of forward and reverse PCR primers.**

Two sources of target DNA were used for the amplification, genomic DNA from cultivar MCol22 and a cDNA library DNA of cassava from cultivar Nga1 constructed in λgt10. While most of the primer combinations either did not produce products or produced products of less than the anticipated minimum sizes, three of the combinations amplified bands of predicted sizes. These were primers 2959f-3745r and 3768f-4003r with cassava genomic DNA **(Figures 4-8, 4.9)** and primers 2176f-2943r with the cDNA library DNA **(Figure 4.10)**. These three PCR products were purified cloned into pGEM-T vector and sequenced. The sequences were edited to remove vector-derived sequence

and were used to search protein databases using BLASTX. BLASTX compares translations of all six reading frames of the input sequence with all non-redundant translations of GenBank, and the protein databases SwissProt, PIR and PRF.

The 860 bp genomic DNA PCR product from the 2959f-3745r primers showed low similarity to several *Arabidopsis* sequences, which included a putative retroelement pol polyprotein (AP001366) and an EST with similarity to a tobacco retroviral-like transposon (AB026295). The 270 bp genomic DNA PCR product from 3768f-4003r primers showed low similarity to catalase from maize (A55092) and *Campylobacter jejuni* (X85130), and a probable L-rhamnose isomerase from *Salmonella typhimurium* (X57299).

The 780 bp cDNA library product from the 2176f-2943r primers only showed low similarity to an *E. coli* hypothetical protein (AE000341). None of the clones contained any xanthine oxidase sequences. The lack of success to clone XO was probably due to the degree of degeneration of the primers used. While alternative strategies might ultimately have yielded the desired results, time constraints prevented further investigation of XO.

| Primer set | Minimum anticipated band size (bp)* |
|---|---|
| 139f - 1466r | 1327 |
| 139f - 1469r | 1330 |
| 139f - 2943r | 2804 |
| 139f - 3745r | 3606 |
| 139f - 4003r | 3864 |
| 459f - 1466r | 1007 |
| 459f - 1469r | 1010 |
| 459f - 2943r | 2484 |
| 459f - 3745r | 3286 |
| 459f - 4003r | 3544 |
| 557f - 1466r | 909 |
| 557f - 1469r | 912 |
| 557f - 2943r | 2386 |
| 557f - 3745r | 3188 |
| 557f - 4003r | 3446 |
| 1563f - 2943r | 1380 |
| 1563f - 3745r | 2182 |
| 1563f - 4003r | 2440 |
| 2176f - 2943r | 767 |
| 2176f - 3745r | 1569 |
| 2176f - 4003r | 1827 |
| 2959f - 3745r | 786 |
| 2959f - 4003r | 1044 |
| 3768f - 4003r | 235 |

**Table 4.1 Xanthine oxidase degenerate primers and minimum anticipated band sizes.**

**f**: Forward and **r**: Reverse primers.

*The size of the band can be larger than the minimum predicted due to the presence of introns.

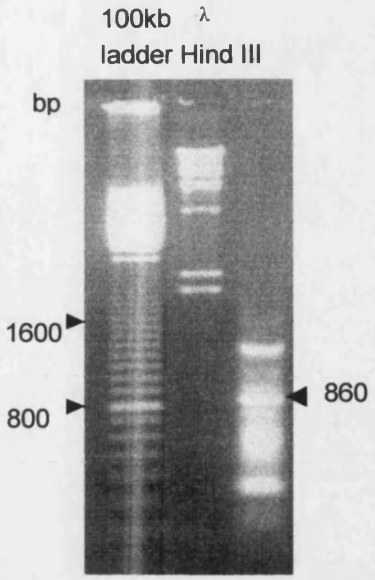



**Figure 4.8 PCR amplification of cassava genomic DNA with 2959f and 3745r.**

**bp**: Base pairs; **Lane 1:** DNA sample amplified at 63°C annealing temperature. The 860 bp band that was cloned and sequenced is indicated.

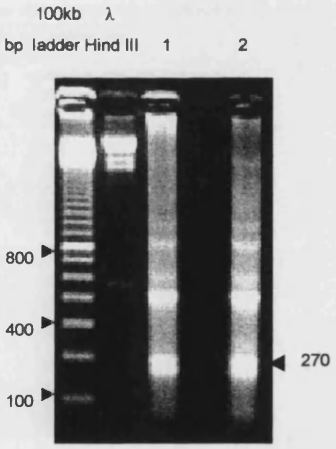



**Figure 4.9 PCR amplification of cassava genomic DNA with 3768f and 4003r.**

**bp:** Base pairs; **Lane 1**: 55°C annealing temperature; **Lane 2:** 59°C annealing temperature. The 270 bp band that was cloned and sequenced is indicated.

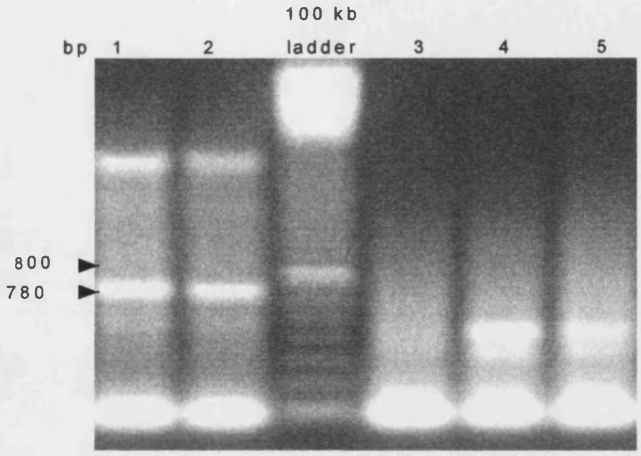



**Figure 4.10 PCR amplification of cassava lambda genomic library and genomic DNA with 2176f and 2943r.**

**bp**: Base pairs; **Lane 1**, **2** were samples obtained from the lambda genomic library of cassava; **Lane 3**, **4** were samples obtained from genomic DNA and amplified from the same set of primers. The 780 bp band that was cloned and sequenced is indicated.

## 2.3 PEROXIDASE ENZYME ACTIVITY

Peroxidase (E.C. 1.11.1.7) is an ubiquitous enzyme that reduces hydrogen peroxide in the presence of an electron donor (Moerschbacher 1992). Most higher plants have a number of peroxidase (POD) isoenzymes with different cellular and tissue localization that are usually classified as anionic, neutral and cationic, based on their isoelectric points (Barz et al. 1990). Peroxidases can be found in cell walls and vacuoles. Cell wall peroxidases are involved in several plant defence mechanisms such as oxidative burst processes (Robertson et al. 1999), lignification and suberization (Carpin et al. 1999; Cochrane et al. 2000), and cross-linking of cell wall components

(formation of isodityrosine and diferulate) (Bernards et al. 1999). Vacuolar peroxidases are involved in tannin condensation, lignin formation and oxidative browning reactions of wounded tissues. Peroxidase activity often increases after wounding, disease infection, growth and elicitation (Cortelazzo et al. 1996; Mcghie et al. 1997; Mensen et al. 1998; Retig 1974).

Increase of POD in infected plants in contrast to the often localized growth of pathogens suggests that an increase in peroxidase activities is triggered in the host plant by the invading pathogen (Moerschbacher 1992). The involvement of this enzyme in different processes that are components of plant defence responses and activity during post-harvest physiological deterioration in cassava reflects the importance of this enzyme during stress responses in this plant. Increases in peroxidase activity during incompatible plant-pathogen/elicitor interactions are often associated with a progressive incorporation of phenolic compounds into the cell wall (Graham 1991; Milosevic & Slusarenko 1996). Evidence from bean suspension cells challenged with *C. lindemuthainum* elicitor suggested the cell wall peroxidases can be a principal source of ROS during the oxidative burst (Robertson et al. 1995). Therefore, determining POD levels, forms and locations in cassava suspension cells challenged with cell wall glucan from yeast was undertaken as part of the investigation of the source of ROS and as a possible modifier of antimicrobial phenolics.

The results suggest that in cassava suspension cells activation of defence responses were triggered by yeast elicitor, which was reflected in the increase of cell wall associated peroxidases. Increase of these peroxidases up to 4-fold and peaked at 48 h was observed in elicitor-treated cassava cells **(Figure 4.11)**. In contrast the extracellular medium contained lower POD activity after 12 h in comparison to the control medium, in which activity increase from 0 to 48 h after the onset of the experiment **(Figure 4.12)**.

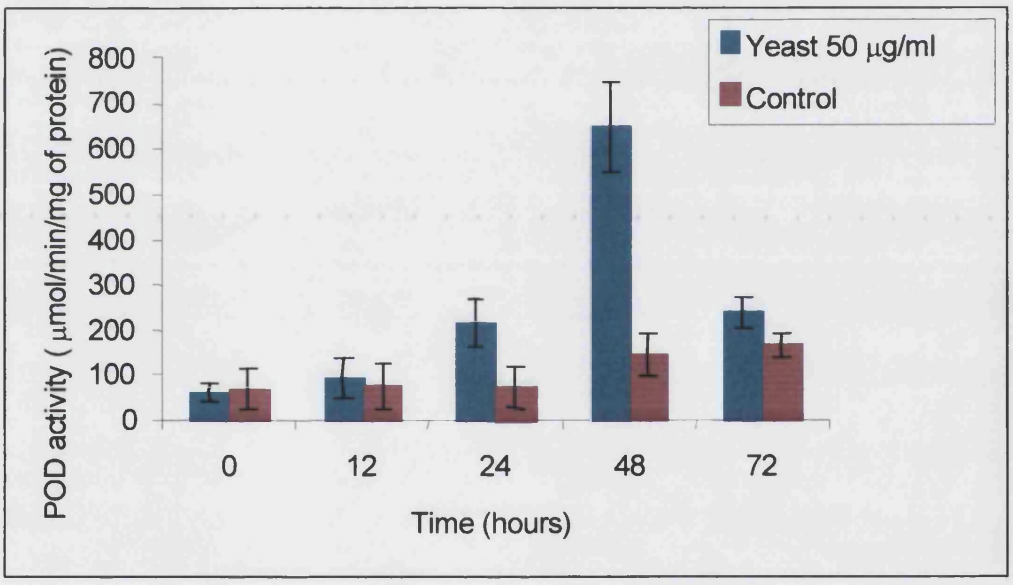



**Figure 4.11 Cell wall-associated peroxidases in elicited and control cassava cells**.

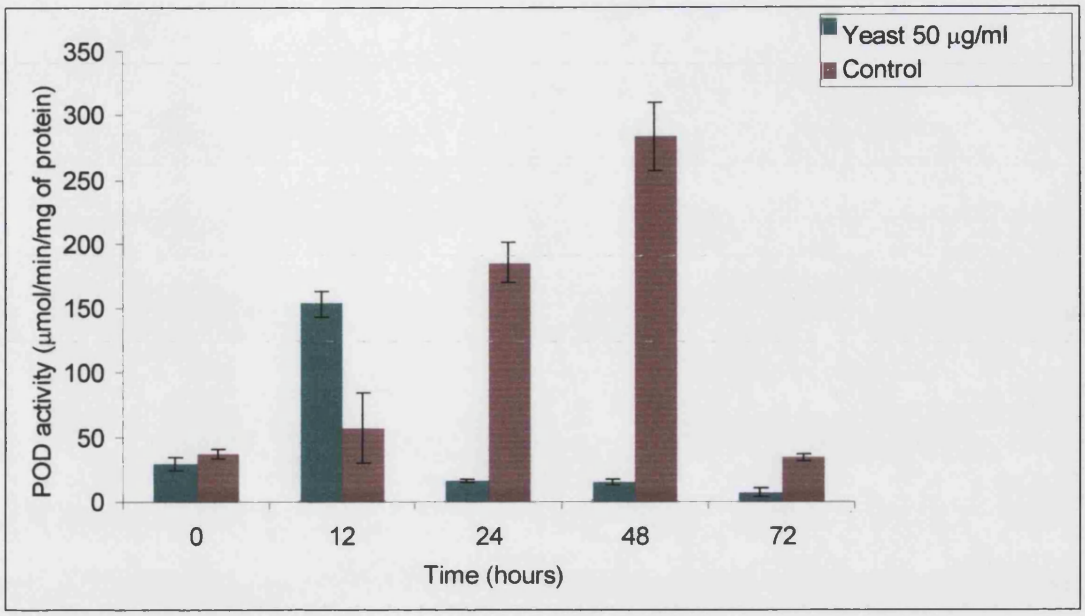



**Figure 4.12 Extracellular peroxidases in elicited and control cassava medium.**

Error bars represent standard error of three independent experiments.

However, extracellular medium while it showed a brief early peak of peroxidase activity at 12 h in response to elicitation, over time (up to 48 h) showed an inhibition of peroxidase activity with respect to the control. These changes in peroxidase activity in response to elicitation may be due to the differential induction, activation or inactivation of peroxidase isoforms. Therefore, an attempt was made to visualize peroxidase isoforms of cassava cells, and the culture fluids and leaves after their challenge with yeast elicitor.

Different peroxidase isoforms were revealed from the three locations **(Figure 4.13)**. The peroxidase enzyme activity showed an increase in elicited cells after 48 h which contrasts with the IEF gel in which elicited cells did not present any difference with the control cells at this time. Obviously, enzyme activity measures total enzyme and it does not distinguish isoforms. Suspension cells

contained 3-4 peroxidase isoforms. However, there was no discernible difference in either isoform number or their intensity between control or elicited cells. At least seven isoforms were detected in the extracellular medium. Two isoforms (pI 3.4 and 8.8) were enhanced in the elicited medium compared to the control. Only two peroxidase isoforms were detectable in leaves and no difference was evident between the elicited and control leaves. One isoform (pI 3.6) was present in all the samples.

Peroxidases are able to oxidize a wide range of organic compounds using hydrogen peroxide as the ultimate electron acceptor (Dawson 1988). For instance, in tobacco suspension cells a particular isoperoxidase $A_3$ was isolated and associated with the oxidation of scopoletin (Reigh et al. 1975). Furthermore, in cassava roots the increase in peroxidase activity has been associated with the increase of some phenolic compounds including scopoletin (Wheatley & Schwabe 1985), and scopoletin is a major phenolic compound found in leaves (see Chapter 5). Therefore, peroxidase isoforms from cassava suspension cells and leaves were tested for their capacity to oxidize scopoletin by soaking a gel in which cassava extracts had been separated by isoelectric focusing in a solution containing hydrogen peroxide and scopoletin **(Figure 4.14)**.

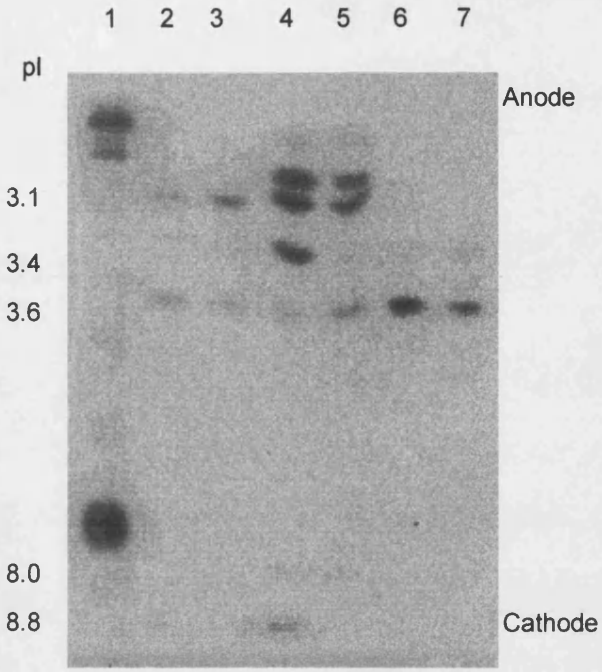



**Figure 4.13 Peroxidase isoforms (by isoelectric focussing) of cassava cells, medium and leaves at 48 h after yeast elicitation.**

Approximately, 5 μg of cassava protein in 20 μl of extraction buffer was loaded into each lane. **Pi**: Isoelectric point; **Lane 1**: Horseradish peroxidase II (positive control); **Lane 2** Cassava elicited cells (48 h); **Lane 3**: Cassava control cells; **Lane 4**: Extracellular medium from elicited cells; **Lane 5**: Extracellular control medium; **Lane 6**: Cassava elicited leaves; **Lane 7**: Cassava control leaves (SDW-treated).

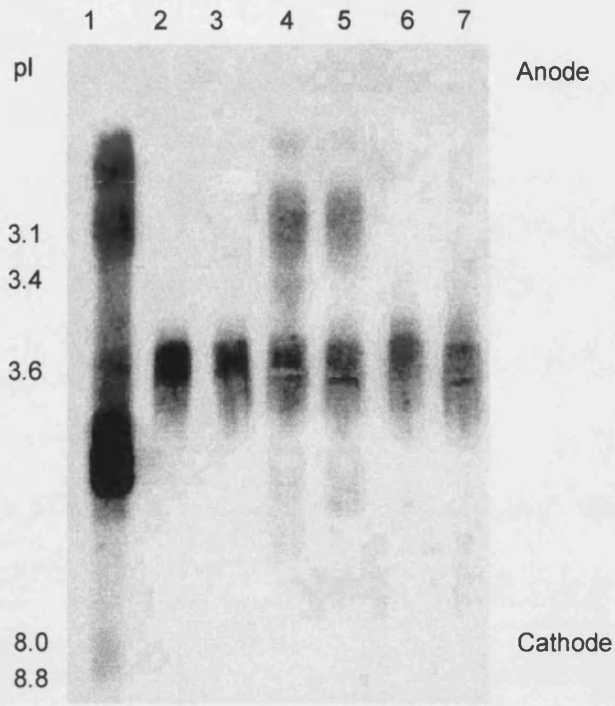



**Figure 4.14 Cassava peroxidase isoforms with activity toward scopoletin.**

**Pi**: Isoelectric point; **Lane 1**: Horseradish peroxidase II (positive control); **Lane 2** Cassava elicited cells (48 h); **Lane 3**: Cassava control cells; **Lane 4**: Extracellular medium from elicited cells ; **Lane 5**: Extracellular control medium; **Lane 6**: Cassava elicited leaves; **Lane 7**: Cassava control leaves (SDW-treated).

**Figure 4.14** shows that all isoforms of horseradish peroxidase were able to oxidize scopoletin to a blue product which later turned yellow. Anionic peroxidase isoforms in particular were able to oxidize scopoletin. In particular, isoform (pI 3.6), which was present in all samples including leaves, was highly effective in scopoletin oxidation.

## 2.4 GENE EXPRESSION

Peroxidase changes during the time course of elicitation may derive from *de novo* synthesis of one of several isoforms. Therefore, it was of interest to investigate whether the corresponding gene(s) were activated. Profiles of the accumulation of corresponding mRNAs during the time course of elicitation were obtained by probing Northern blots of total RNA with cassava cDNA probes. In addition to peroxidase, other clones were used, namely catalase and HRGP as these proteins are either involved in the modulation of ROS or require ROS **(Figure 4.15)**. It must be pointed out that Northern blots measure the abundance of a particular mRNA species, which is on equilibrium between its rate of synthesis and degradation. While Northern blots are often interpreted as measuring gene expression, they in fact measure mRNA accumulation; nuclear run-on experiments are required in order to give definitive measures of gene expression.

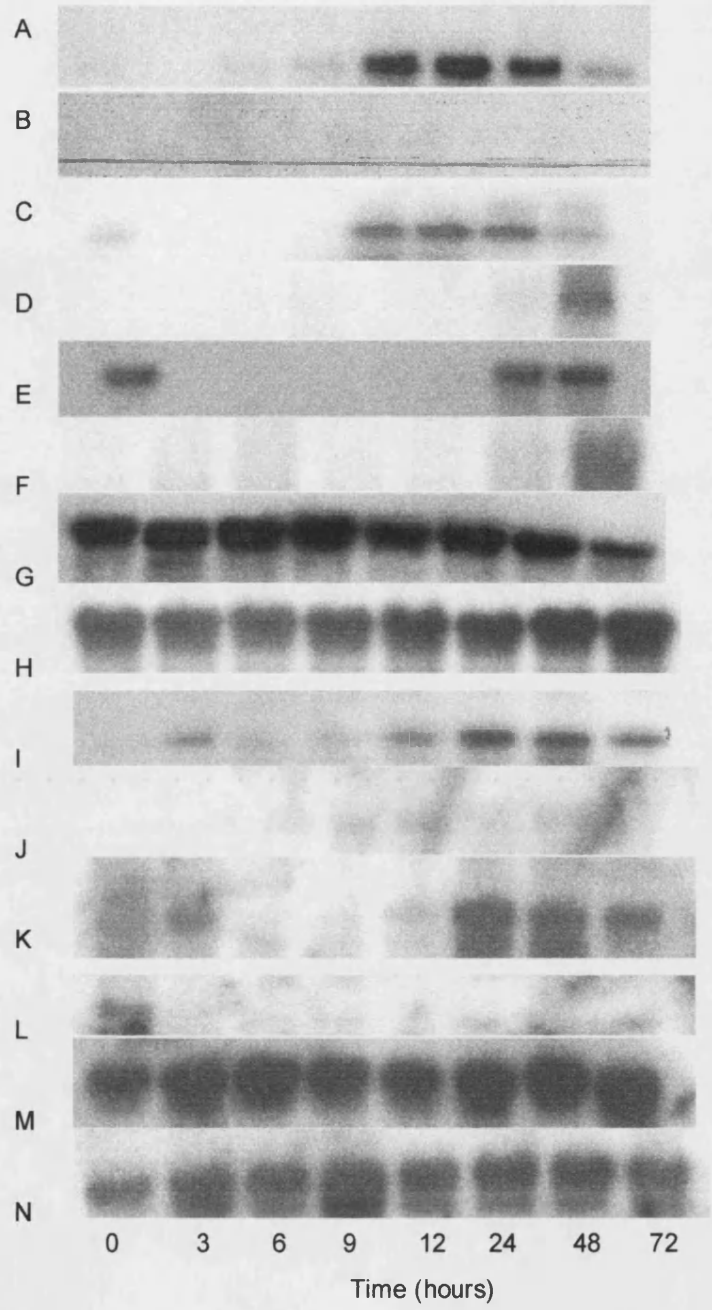



**Figure 4.15 Gene expression in cassava suspension cells and leaves after elicitation with yeast cell wall glucan.**

Northern blots of cassava RNA purified after elicitation and probed with cassava POD1, HRGP1 and Catalase1 cDNA clones. 10 μg of total RNA loaded per lane. **A**: POD in elicited cells; **B**: POD

in control cells; **C**: HRGP in elicited cells; **D**: HRGP in control cells; **E**: CAT in elicited cells; **F**: CAT in control cells; **G**: 18SrDNA for elicited cells; **H**: 18SrDNA for control cells; **I**: POD in elicited leaves; **J**: POD in control leaves; **K**: CAT in elicited leaves; **L** CAT in control leaves; **M** 18SrDNA for elicited leaves; **N**: 18SrDNA for control leaves.

**Figure 4.15** shows different Northern blots obtained with cassava cells and leaves after yeast elicitor challenge. Expression of the MecPOD1 started 12 h after elicitation in cells. Comparing this result with that for peroxidase activity in cells which was maximal at 48 h after elicitation, suggests that elicitor stimulates mRNA synthesis of this transcript in cassava cells. Additionally, no expression was observed in control cells, which contrasts with the results obtained for peroxidase enzyme activity, suggesting that this activity is due to expression of other peroxidase genes. Furthermore, MecPOD1 started to be induced 3 h after elicitation in leaves, suggesting that elicitation and wounding were responsible for this expression.

Expression of MecHRGP in elicited cells was similar to that of MecPOD, with transcript accumulation detected after 48 h following elicitation. In control cells transcript expression was unexpectedly observed at 72 h. A possible explanation may be that at 72 h cell were in stationary phase and the expression observed may reflect generalised stress responses.

With regard to catalase, MecCAT1 was present in cassava suspension cells initially and was down regulated after elicitation, although expression had recovered by 24 and 48 h. In leaves, MecCAT was expressed until 3 h after the onset of elicitation and was down regulated until 24 h when increased expression occurred until 72 h. These results may explain why catalase enzyme activity was

not detected in elicited or control cells. Again, expression was observed in control cells at 72 h only which may be its due to cells entering into stationary phase.

## 2.5 NON ENZYMATIC ANTIOXIDANTS IN CASSAVA SUSPENSION CELLS AND LEAVES AFTER YEAST ELICITOR CHALLENGE

Cells use substantial resources to protect themselves from the potentially damaging effects of reactive oxygen species. Several vitamins and micronutrients, which are active at quenching these free radical species or are required for their enzymatic detoxification, as well as enzymes, such as superoxide dismutase (SODs), glutathione peroxidase (GPOD), and catalases (CATs), constitute the first line of defence against ROS, and are generally referred to as primary antioxidants (Cadenas 1995). Non-enzymatic antioxidant compounds have also been detected in plants, such as catechin, catechin gallate, rutin, quercetin-3-O-glucoside, quercetin-3-O-rhamnoside, and kaempferol-3-O-rutinoside (Lohman et al. 1994)(Thabrew, 1998).

HP-TLC plates were used to analyse antioxidant properties of extracts from cassava cells and leaves after challenge with yeast elicitor. **Figure 4.16** shows that cassava leaves and suspension cells (elicited and control) contained antioxidant compounds at all time points. Cassava leaf samples (yeast elicited) after 12 h to 96 h showed an increase in the quantity of antioxidant compounds **(Lane 5-7)**.

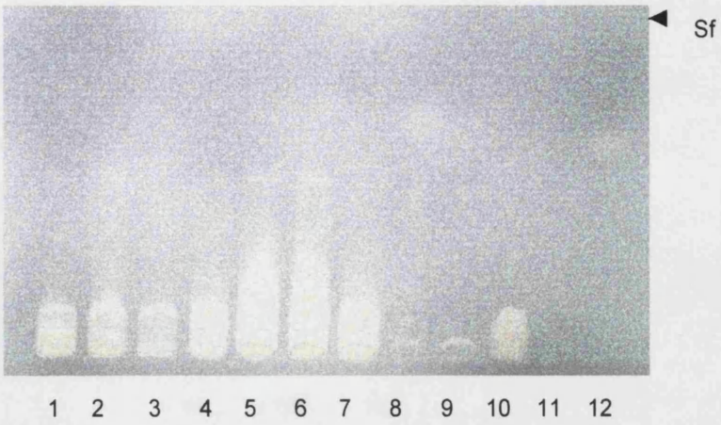



**Figure 4.16 HP-TLC plate with methanolic extracts from cassava cells and leaves stained for detection of antioxidants with DPPH compound.**

**Sf**: Solvent front; **Lane 1**: Cassava leaves at 0 h (unelicited); **Lane 2**: Cassava leaves at 48 h (unelicited); **Lane 3**: Cassava leaves after 12 h (yeast elicited); **Lane 4**: Cassava leaves after 24 h (yeast elicited); **Lane 5**: Cassava leaves after 48 h (yeast elicited); **Lane 6**: Cassava leaves after 72 h (yeast elicited); **Lane 7**: Cassava leaves after 96 h (yeast elicited); **Lane 8**: Cassava suspension cells after 48 h (yeast elicited); **Lane 9**: Cassava suspension cells after 48 h (unelicited); **Lane 10**: Rutin commercial standard; **Lane 11**: Kaempferonl-3-rutinoside commercial standard; **Lane 12**: Scopoletin commercial standard.

**Figure 4.16** showed that cassava leaves contained abundant antioxidant compounds. The quantity of antioxidants increased after 24 h in control and elicited leaves with a decrease at 72 h. Rutin showed antioxidant activity, but kaempferol-3-*O*-rutinoside and scopoletin did not. Compounds were not separated by this system thus no attempt was made to identify them.

## 3 DISCUSSION

A rapid transient production of high amounts of ROS, is one of the earliest observable aspects of a plant's defence strategy (Bolwell et al. 1999). Elicitors from a wide range of sources were tested with cassava suspension cells. However, cell wall glucan elicitors appeared to be the most efficient at generating a oxidative burst and also the source was readily available and the preparation facile. Cassava suspension cells treated with yeast elicitor responded with an increase in the production of extracellular $H_2O_2$ of 10.5 μM. These results were similar to those observed in soybean suspension cells treated with oligalacturonide elicitor (Chandra & Low 1995). Only three other elicitors induced significant $H_2O_2$ production; these were cell wall glucan from *C. lindemuthianum*, oligalacturonic acid and glutathione all between 20-30 min after elicitation. These elicitors were previously reported to induce oxidative burst in other plants (Legendre et al. 1993; Robertson et al. 1995; Bolwell & Wojtaszek 1997). This rapid and transient oxidative burst began after 2-3 min, then only one peak occurred with maximum intensity between 20-30 min for all the elicitors tested.

ROS generation can occur in two phases; although the first phase is readily stimulated by purified elicitors, the second one is generated in the interactions with incompatible pathogens (Baker et al. 1993; Legendre et al. 1993). Two peaks were produced in cassava suspension cells treated with the incompatible phytopathogenic bacterium *Erwinia amylovora* ($1 \times 10^7$ cfu/ml) the first one at 30 minutes and the second one started at 80 minutes with a maximum peak at 130 min (R.M. Cooper, personal communication).

A controversial debate exists about the origin and role of the oxidative burst during plant-pathogen interactions. Indeed up to five different mechanisms had been

described in various plant species, including a pH-dependent cell-wall peroxidase, a plasma membrane NADPH oxidase system, germin-like oxalate oxidase, amine oxidases and protoplasmic mitochondrial, peroxisomal, and/or glyoxysomal activities of infected cells. The two dominant models of ROS generation during the oxidative burst which are supported by a large body of evidence from several groups are the plasma membrane NADPH oxidase system and the pH-dependent $H_2O_2$-generating peroxidase (Bolwell et al. 1999; Auh & Murphy 1995). Several inhibitors have been used in attempts to understand the origin of the oxidative burst in plants (Bolwell et al. 1998). Oxygen radicals formed during the respiratory burst in cassava suspension cells challenged with cantharidin, cell wall glucan from yeast and *C. lindemuthianum* elicitors and *X. axonopodis* pv. *manihotis* (*Xam*) (isolate 2967) appeared to originate mainly from the NADPH-dependent enzyme system. High sensitivity to diphenyleneiodonium (a potent inhibitor of the mammaliam NADPH oxidase) and the increased levels of hydrogen peroxide in the presence of superoxide dismutase (SOD) suggested that the generation of $H_2O_2$ proceeded *via* flavoprotein-mediated synthesis of $O_2^-$ (R.M. Cooper personal communication).

ROS production plays several roles, for example extracellularly as antimicrobial agents, at the level of the cell wall in oxidative cross-linking of proteins, intracellularly as signal intermediates triggering changes in gene expression, and possibly as toxin molecules contributing to host cell death (Mehdy 1994). Indeed, $H_2O_2$ produced by elicited plants *in vitro* is sufficient to retard significantly microbial growth (Peng & Kuc 1992).

Xanthine oxidase (XO) has been proposed as a possible source of the oxidative burst in plants (Slusarenko & Milosevic 1995). XO appeared to be constitutive in both elicited and control cells with no apparent increase after

elicitation. This constitutive expression of the enzyme may be due to the fact that suspension cells are under stress conditions. In Red Mexican bean leaves challenged with virulent and avirulent isolates of *Pseudomonas syringae* XO activity increased substantially in HR tissue induced by incompatible race and only slightly in the area inoculated with the compatible race; XO remained unchanged in the surrounding leaf areas (Slusarenko & Milosevic 1995). The attempt to clone cassava XO here were unsuccessful perhaps because only XO genes from animal, insect and fungal species, *Bos Taurus* (B-tau), *Homo sapiens* (H-sap), *Gallus gallus* (G-gal), *Drosophila melanogaster* (D-mel) and *Aspergillus nidulans* (A-nid) were available for the design of PCR primers. The lack of success to clone XO was probably due to the degree of degeneration of the primers used. It is notable that any plant XO has yet to be cloned

Plant peroxidases are ubiquitous enzymes that have been reported to be involved in numerous physiological processes and disorders including wounding, defence, lignification and suberisation of the cell-wall, indole acetic acid (IAA) catabolism, and hydrogen peroxide production (Allan & Fluhr 1997; De Marco et al. 1999; Huh et al. 1997; Moerschbacher 1992; Plumbley et al. 1981; Trevisan et al. 1997). In elicitor-treated cassava suspension cells symplasmic peroxidase activity increased with a peak at 48 h after elicitation and levels were higher than in the extracellular medium. The extracellular activity from elicited cells decreased after 12 h possibly due to inactivation caused by the elicitor or plant factor(s) induced by it. These results were similar to those described in spruce (*Picea abies* L.) suspension cells challenged with *Rhizosphaera kalkhoffii* and slash pine suspension cells challenged with chitosan elicitor (Lesney 1990; Messner & Meinrad 1994).

A cassava peroxidase clone MecPOD1 was previously isolated in this laboratory (Dr. Kim Reilly, personal communication). The clone shows highest similarity up to 65 % with adzuki bean (*Vincula angularis*) cationic peroxidase, but low levels of similarity to a partial cassava genomic peroxidase clone (Dr. L.F. Pereira et al., unpublished) available on the GenBank database.

The elicitor-treated cells showed transcript accumulation from 12 h to 48 h with a peak at 24 h and transcript accumulation of MecPOD1 preceded maximal enzyme activity suggesting induction of gene transcription and *de novo* enzyme synthesis in response to elicitation. In elicitor-treated leaves, low levels of expression were evident from 3 h with maximum accumulation at 24 to 48 h. The low initial levels of transcription may reflect a wounding effect. Transcript levels at 24 and 48 h in leaves were lower than those observed in suspension cells possibly because cells are less heterogeneous than those in leaves.

Peroxidases play a key role in the cross-linking of tyrosine residues in the cell-wall protein extensin (HRGP) (Moerschbacher 1992). Reinforcement of the plant cell wall by these proteins has been considered as a defence mechanism in plants (Allan & Fluhr 1997). Use of a cassava extensin cDNA clone (MecHRGP1) was in Northern analysis of elicitor-treated cassava suspension cells showed a similar pattern of expression to that found with MecPOD1, with transcript accumulation at 12 to 48 h after elicitation.

The increase of peroxidases after infection has often been correlated with an increase or appearance of new isoforms (Chittoor et al. 1997; Ludwig-Müller et al. 1994; Shaul & Elad 1995). Isoperoxidases, arising from the transcription of different genes or from post-translational modification, are widely distributed within both the intra- and the extracellular environment (Zapata et al. 1995). Many different isoforms

have been detected in plants with different cellular and tissue localization (Perrey et al. 1989). Cassava cells, spent medium, and leaves presented a different number of isoforms in the IEF gels. Mainly anionic peroxidases were observed and the isoform pI 3.4 increased markedly post-elicitation. This was in contrast to other plants in which basic isoperoxidases were predominant such as the case of *Lupinus polyphyllus*; furthermore, it was stated that anionic peroxidases were cell wall-bound due to their absence in *L. polyphyllus* protoplasts (Perrey et al. 1989). Specific roles for isoperoxidases have not been fully elucidated (Boeuf et al. 2000). Each isoenzyme is thought to play a different function during plant cell growth and development, although their actual physiological roles in plant physiology still unclear (Kim et al. 2000). When IEF gels were stained with scopoletin and hydrogen peroxide a single isoform pI 3.6 that was present in all the samples tested showed high activity towards scopoletin. Scopoletin has been reported elsewhere to be antimicrobial, it was found in cassava cells and leaves and has been reported before to be present in cassava (see Chapter 5).

Catalase ($H_2O_2$:$H_2O_2$ oxidoreductase EC 1.11.1.6) has been considered as a primary antioxidant defence strategy for plants (Cadenas 1995). Two types of catalase are known such as Mn catalases (prokaryotes) and the more widespread tetrameric heme-containing catalases (Bravo et al. 1997). Catalase occurs as multiple isoenzymes encoded by a small gene family; for instance there are two members in castor bean and five in maize (Suzuki et al. 1995; Williamson & Scandalios 1993). Northern blots using MecCAT1 as probe in elicitor-treated cassava cells showed down-regulation from 3 h after elicitation followed by a recovery in transcription at 24 h. Similarly in elicitor-treated leaves catalase transcript accumulation was down regulated from 3 h following elicitation with recovery at 24 to

48 h. It has previously been suggested that catalase enzyme activity is inhibited by binding to salicylic acid thus allowing increased levels of hydrogen peroxide during SAR (Chen et al. 1993). Results from Northern blotting suggested that in challenged cassava tissues catalase down-regulation at the transcriptional level may play a role in facilitating the formation of hydrogen peroxide. In Red Mexican bean leaves challenged with virulent and avirulent isolates of *Pseudomonas syringae*, catalase activity decreased in incompatible but not in compatible reactions in the inoculated leaf area, but remained unchanged in the surrounding leaf areas for both interactions. The authors identified catalase as a host factor which was specifically down-regulated at the level of transcription and enzyme activity in the necrotizing area undergoing HR (Slusarenko & Milosevic 1995)

Finally, in addition to the enzymatic antioxidants mentioned above (catalase and peroxidase), plants may utilise non-enzymatic antioxidant compounds. These antioxidant activities are mainly associated with a diverse range of phenolic compounds, but principally with flavonoids and proanthocyanidins (Hanasaki et al. 1994; Rakotoarison et al. 1997; Saija et al. 1995). The presence of flavonoids including rutin and quercetin has been reported in cassava (Calatayud et al. 1994). Cassava extracts were separated by HPTLC plates and stained with the free radical DDPH. Higher levels of antioxidant compounds appeared in elicited leaves compared with cells. In addition a peak occurred in elicited-treated leaves after 48 h. Rutin, kaempferol-3-*O*-rutinoside and scopoletin, were detected in cassava cells and leaves. Rutin but not kaempferol-3-*O*-rutinoside showed strong antioxidant activity and functioned as a strong antioxidant in the cassava extracts. The scopoletin standard showed strong antioxidant activities, however it did not function as a strong antioxidant in the cassava extracts tested.

# CHAPTER 5

# PHENYLPROPANOID METABOLISM IN ELICITOR-CHALLENGED CASSAVA

## 1 INTRODUCTION

An ubiquitous feature of plant responses to pathogen or elicitor challenge is the activation of phenylpropanoid metabolism. Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) catalyses the first committed step of the core pathway of general phenylpropanoid metabolism, branch pathways from which lead to the synthesis of compounds that have several different functions in plants. For instance, cell wall strengthening and repair (e.g. lignin and suberin), antimicrobial activity (e.g. furanocoumarins and isoflavonoids), UV protectants (e.g. flavonoid pigments), signaling compounds (e.g. salicylic acid) (Diallinas & Kenellis 1994; Dixon et al. 1994; Hammerschmidt 1999; Durner et al. 1997; Yalpani et al. 1994). However, while PAL diverts phenylalanine from primary to secondary metabolism and controls its flux into general phenylpropanoid metabolism, the accumulation of individual products depends on the activities of those enzymes that catalyse the different branch pathways. In cassava, induction of PAL has been observed during post-harvest physiological

deterioration (PPD), wounding and in response to *Xam* (Pereira et al. 1999; Rickard 1981; Tanaka et al. 1984), but has not been studied so far in elicited suspension cells.

Since the 1950s, numerous studies have revealed the accumulation and synthesis of various secondary metabolites including phenolics in plants. As early as 1935 it was reported that onion varieties resistant to *Colletotrichum circinans* accumulated water soluble phenolic compounds in their bulb scales that inhibited the germination and penetration of the pathogen (Walter & Link 1935). Induction of PAL has been linked to the subsequent *de novo* production of phytoalexins in many plants species (Hammerschmidt 1999; Dixon et al. 1983). In particular, yeast elicitor has been reported to induce PAL and the consequent accumulation of phytoalexins and other secondary metabolites in different plants including alfalfa, tobacco, *Lupinus albus*, apple, *Eschscholtzia californica* (Californian poppy), *Solanum khasianum*, and soybean (Baier et al. 1999; Borejsza-Wysocki et al. 1999; Fahrendorf et al. 1995; Guo et al. 1998; Mühlenbeck et al. 1996; Roos et al. 1998; Wojtaszek & Stobiecki 1997). By contrast, other reports showed that phenolic compounds are not necessarily associated with resistance. For instance, a drop in phenolic levels in tomato caused by high nitrogen fertilization is accompanied by an increased resistance against infection caused by *F. oxysporum* (Sarhan et al. 1982).

The *Euphorbiaceae* family is rich in flavonoids, particularly flavones and flavonols. They occur as both *O*- and *C*-glycosides and as methyl ethers. Flavonoids are detected in different parts of the plant including roots. Two common flavonoids, kaempferol and quercetin (and their glycosides), are the most widely distributed in different genera of this family (Fattah & Rizk 1987).

In order to ascertain the effects of yeast elicitation on phenylpropanoid metabolism in cassava suspension cells, the activity of PAL and its genes, together with the accumulation and activity of phenylpropanoid products were determined. To confirm that observations made in the cell/elicitor model system were applicable to tissues, many of these experiments were duplicated *in planta* using leaves.

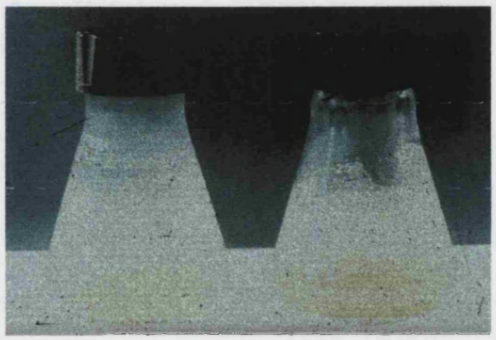
## 2 RESULTS

### 2.1 ELICITATION OF PHENYLALINE AMMONIA LYASE ACTIVITY AND TRANSCRIPTS

An increase of phenolic compounds was suspected after the coloration of the cassava cells changed from transparent-yellow to brownish-yellow once cells were exposed to yeast elicitor for at least 12 h and this colour became darker until 24h **(Figure 5.1)**. These changes were not due to cell death induced by yeast elicitor as growth appeared unaffected **(Figure 5.2)**.

Alkalinization of the medium, an oxidative burst and peroxidase induction in cassava cells were observed previously with yeast elicitor (50 μg/ml) (Chapters 3-4). However, these are often a dose-dependent plant responses to elicitors, and even in the same plant different enzymes can be activated with different elicitor concentrations (Dixon et al. 1981; Hahlbrock et al. 1981; Kombrink & Hahlbrock 1986). Therefore, in order to select the concentration of yeast elicitor optimal for PAL induction in cassava cells, PAL mRNA accumulation in response to a range of elicitor concentrations (5-500 μg/ml) was determined by Northern blottling.

Maximum PAL mRNA accumulation occurred with 50 μg/ml of yeast elicitor **(Figure 5.3)**. 50 μg/ml was selected and used in all subsequent experiments.



A B

**Figure 5.1 Browning in cassava cell suspensions at 12 h after yeast elicitation.**

**A**: SDW-treated control cells; **B**: Elicited cells with yeast elicitor. The flasks contained 50 ml of cassava suspension cells.

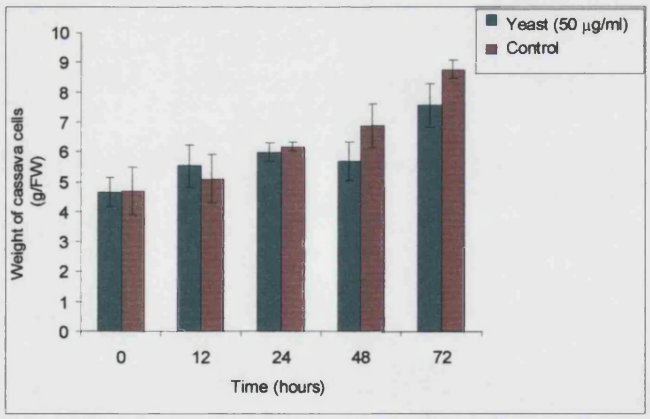



**Figure 5.2 Growth of elicited cassava cells.**

Error bars represent standard deviation of three independent experiments. Weight of cassava cells in grams per fresh weight per flask.

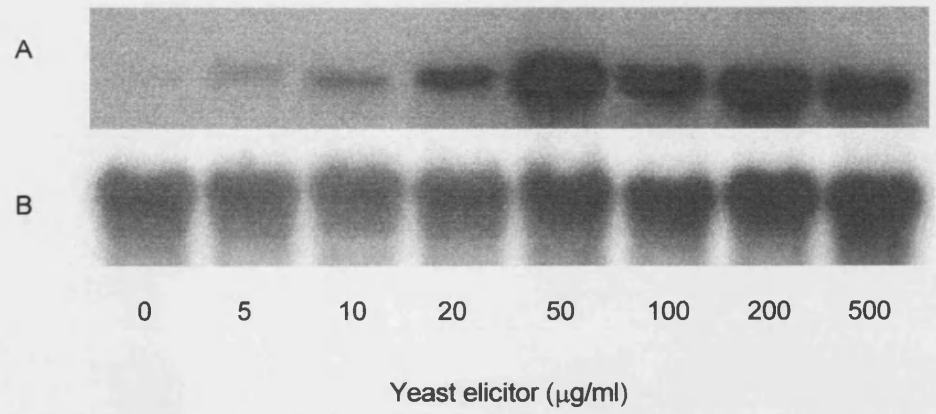



**Figure 5.3 Dose-dependent induction of PAL mRNA.**

Northern blot of cassava cell suspension RNA purified 3 h after elicitation and probed with cassava PAL1 cDNA clone. **A**: Elicited cells; **B**: 18SrDNA to check equal loading. All lanes were loaded with 10 μg of total RNA. RNA size-markers on the original gel indicated a size of ~ *ca.* 2.5 kb for the PAL mRNA.

In order to investigate the relationship between PAL enzyme activity and mRNA accumulation cassava cell suspensions were elicited with yeast at 50 μg/ml. **Figure 5.4** shows temporal increases of PAL activity in yeast-treated cells with up to a 4-fold increase relative to the control values. The maximum peak occurred at 15 h after elicitation. This rapid increase of PAL activity from very low basal levels suggested that elicitor stimulates mRNA synthesis in cassava suspension cells. This was confirmed by the Northern blot in which an accumulation of PAL mRNA was detected within 3 h of treatment and peaked at 9 h **(Figure 5.5)**. These results show similar profiles over time of changes in PAL activity and mRNA accumulation. However, the mRNA peak preceded that of enzyme activity by a few hours, suggesting that the increases in PAL activity were due to increases in expression of the corresponding PAL gene(s). The rise and fall of both PAL mRNA and enzyme activity within a 24 h timescale imply that both have a limited half-life.

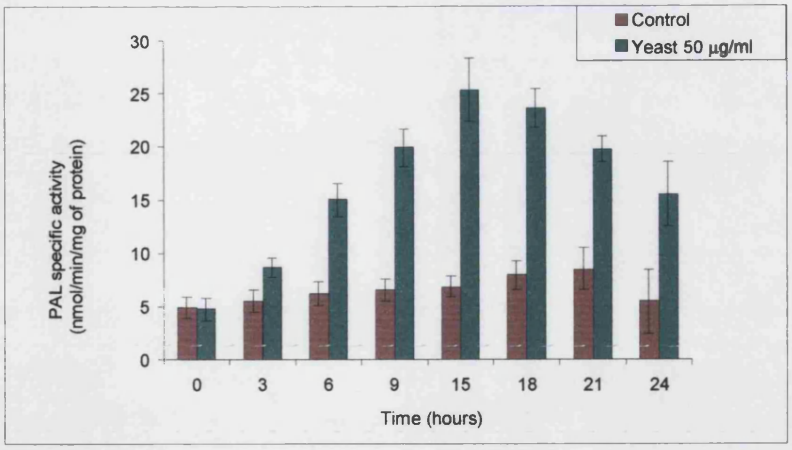



**Figure 5.4 Changes in PAL specific activity in elicited cassava cells.**

Error bars represent standard error of three independent experiments.

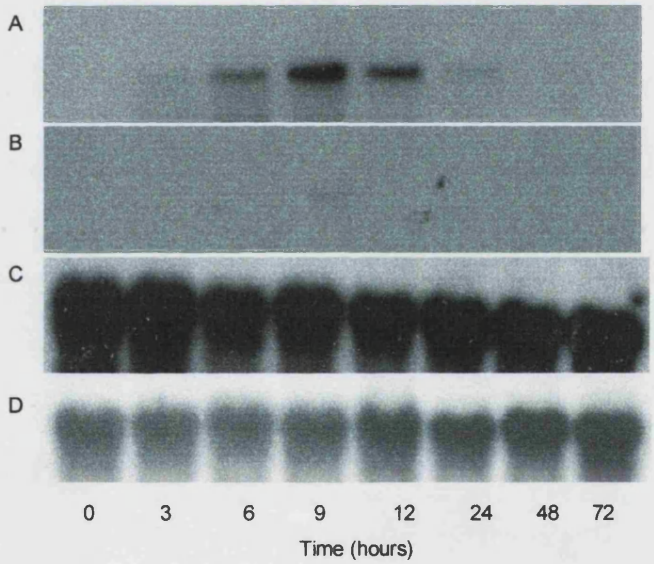



**Figure 5.5 Elicitation of PAL mRNA in elicited cassava cells.**

**A**: Elicited cells with yeast elicitor (50 µg/ml); **B**: SDW-treated control cells; **C**: 18SrDNA to check equal loading in elicited cells; **D**: 18SrDNA to check equal loading test in control cells. All lanes were loaded with 10 µg of total RNA.

These results also suggest that the elicitor activation of PAL genes was rapid. In order to investigate this, Northerns covering a time course of up to 180 minutes were probed for PAL mRNA **(Figure 5.6)**. These results confirmed that

the elicitation of PAL gene activity was indeed rapid, occurring within 30 minutes of elicitation. The surprising detection of PAL mRNA at 10 minutes in the control cells is not readily explicable. Unfortunately, no replicate Northern blot experiments were performed to confirm the presence of PAL mRNA at this time point.

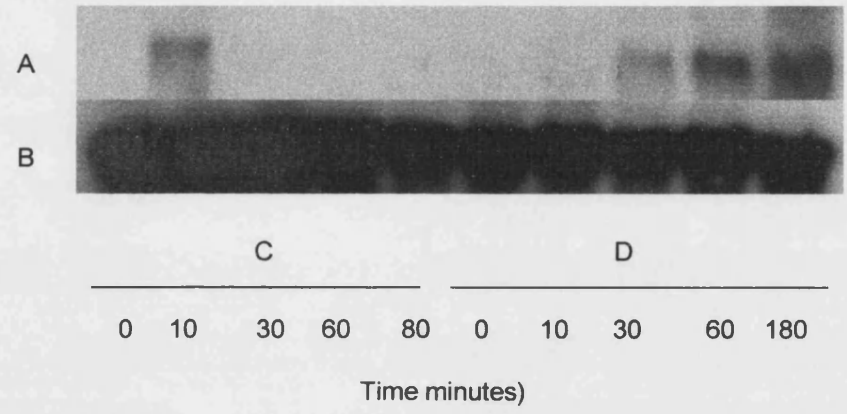



**Figure 5.6 Early induction of transcription of PAL mRNA.**

**A**: Elicited and control cells over time course; **B**: 18SrDNA to check equal loading; **C**: SDW-treated control cells; **D**: Elicited cells with yeast elicitor (50μg/ml). All lanes were loaded with 10 μg of total RNA.

## 2.2 POTENTIATION OF PAL EXPRESSION BY SA PRE-TREATMENT

Salicylic acid is a wound and pathogen-related plant signal molecule, which is also a product of a branch pathway of phenylpropanoid metabolism. Salicylic acid can potentiate stress responses induced by other factors and plays a key role in systemic acquired resistance (Thulke & Conrath 1998). Therefore, cassava suspension cells were pre-treated with a range of concentrations of salicylic acid for 24 h and then elicited with two different

concentrations of yeast elicitor for 3 h. Northern blots of total RNA were then probed for PAL mRNA **(Figure 5.7)**.

At both elicitor concentrations there was a large potentiation of response by salicylic acid pre-treatment, at 150-500 μM salicylic acid with 5 μg/ml elicitor and 150-250 μM with 50 μg/ml elicitor. It is particular interesting that 5 μg/ml yeast elicitor concentration, which on its own elicited barely detectable PAL mRNA levels **(Figure 5.3)**, elicited a substantial response when the cells were pre-treated with salicylic acid **(Figure 5.7)**. However, the responses of the pre-treated cells were very similar at both concentrations of elicitor.

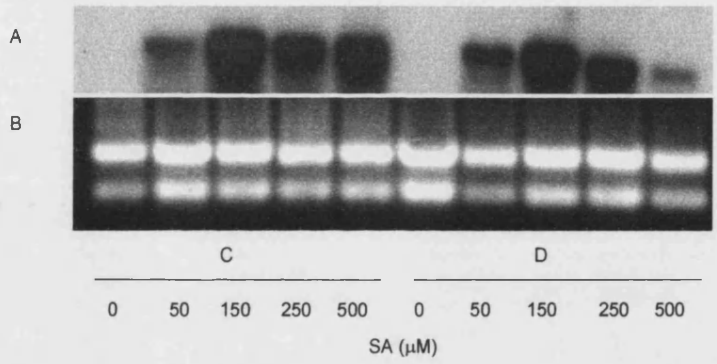



**Figure 5.7 Potentiation of elicited PAL gene activation by SA pre-treatment**

**A**: Elicited cassava cells; **B**: Ethidium bromide stained gel to check equal loading; **C**: Elicited cassava cells with 5 μg/ml yeast elicitor; **D**: Elicited cassava cells with 50 μg/ml yeast elicitor. All lanes were loaded with 10 μg of total RNA purified from cells that had been pre-treated with salicylic acid for 24 h followed by 3 h of elicitation with yeast.

## 2.3 PHENOLIC COMPOUNDS AND OTHER SECONDARY METABOLITES IN CASSAVA CELLS AND LEAVES

The induction and activity of PAL, together with the colour change observed in elicited cells, suggested that phenolic secondary metabolite

production was induced during elicitation. Terpenoids and flavonoids have been already identified in cassava root and leaf tissues in response to post harvest physiological deterioration and insect attack (Sakai & Nakagawa 1988). Therefore, specific stains were used in order to identify different classes of metabolites on HPTLC plates on which were separated extracts from cassava cell suspensions and leaves.

Folin Ciocalteu reagent was used to detect phenolic compounds in order to visualize any possible change in these compounds. **Figure 5.8** shows a typical HPTLC plate with separation of at least 17 bands in elicited cells and 15 bands in controls cells. Two novel bands with Rfs of 0.72 and 0.84 were detected in the elicited cell extracts after 48 and 72 h, the former fluoresced blue under UV light of $254_{nm}$. These data indicated at least an increase in number, if not amount, of phenolic compounds in cassava cells in response to elicitation.

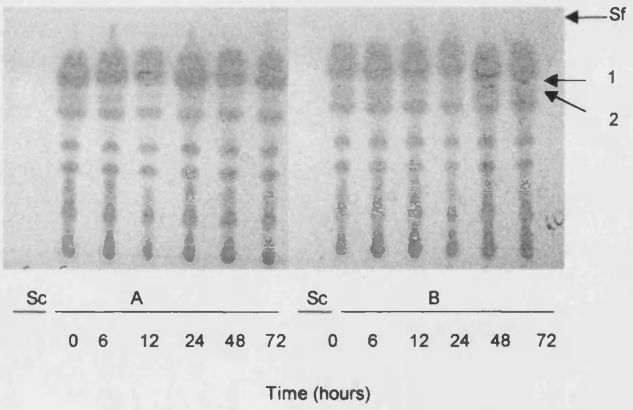



**Figure 5.8 Detection of phenolic compounds from elicited cassava cells.**

HPTLC-separated cassava cell extracts stained with Folin Ciocalteu reagent. **A**: SDW-treated control cells; **B**: Cassava elicited cells with yeast elicitor (50μg/ml); **Sf**: Solvent front (Sf = 5.6 cm); **Sc**: Solvent control. All samples were 250 mg of cells in 40μl methanolic extract applied as spots to HPTLC plates; **1**: Band in cassava cells at 48 and 72 h after elicitation with Rf = 0.72; **2**;

Band in cassava cells at 48 and 72 h after elicitation with Rf = 0.642. Bands were visualized by the stain reagent and under 254 and $366_{nm}$. Rfs for all other bands shown are given in **Appendix 3**.

Terpenoids have been detected in deteriorating cassava roots (Sakai et al. 1986). Therefore, in order to ascertain whether similar compounds were synthesised in elicited cassava cells, Ehrlich's reagent was used for terpenoid detection **(Figure 5.9)**. The HPTLC plates showed an increase in number and abundance of terpenoid- type compounds in elicited cassava cells compared to control cells. In particular, an increase was observed in bands with Rf = 0.48 and Rf = 0.52 between 48 and 72 h after elicitation. These two bands were only visualized with Ehrlich's reagent under $366_{nm}$. Unfortunately, the terpenoid compounds reported previously in cassava were not available as references and commercial compounds were prohibitively expensive, so it was not possible to confirm whether the same stress induced compounds found in roots were also present in elicited cells.

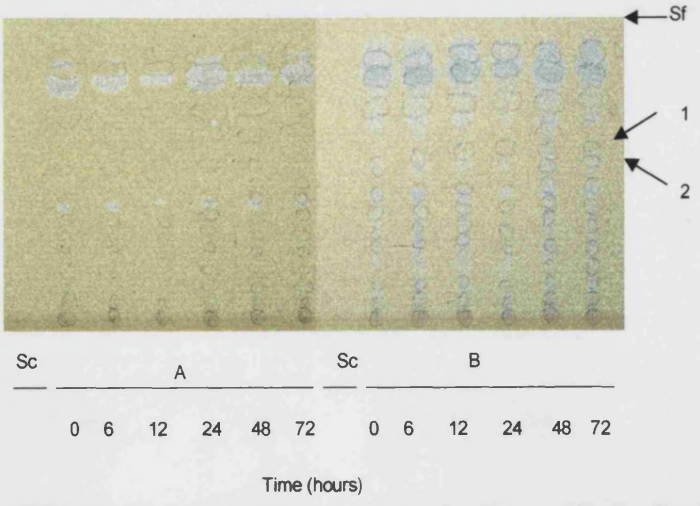



**Figure 5.9 Detection of terpenoid compounds from elicited cassava cells.**

HPTLC-separated cassava cell extracts stained with Erhlich's reagent. **A**: SDW-treated control cells; **B**: Cassava cells with yeast elicitor (50μg/ml); **Sf**: Solvent front (Sf = 5.6 cm); **Sc**: Solvent

control. All samples were 250 mg of cells in 40µl methanolic extract applied as spots to HPTLC plate; **1**: Band in elicited cassava cells at 48 and 72 h after elicitation with Rf = 0.48; **2**: Band in elicited cassava cells at 48 and 72 h after elicitation with at Rf = 0.52. Bands were visualized by the stain reagent and under 254 and $366_{nm}$. Retention factor for all bands shown are given in **Appendix 4**.

Coumarins and flavonoids have been detected in cassava root stress responses and leaves after insect attacks (Calatayud et al. 1994; Wheatley & Schwabe 1985). Therefore, in order to investigate their presence in cassava cell cultures and leaves in response to elicitation extracts from cassava cells and leaves were separated by HPTLC together with references of scopoletin, its conjugated derivative scopolin (scopoletin-7-o-glucoside) (kindly provided by Dr Goro Taguchi, Shinshu University, Japan), rutin and kaempferol-3-rutinoside, and stained with Neu reagent to visualize coumarins and flavonoid compounds present in cassava extracts under UV $366_{nm}$ **(Figures 5.10, 5.11)**.

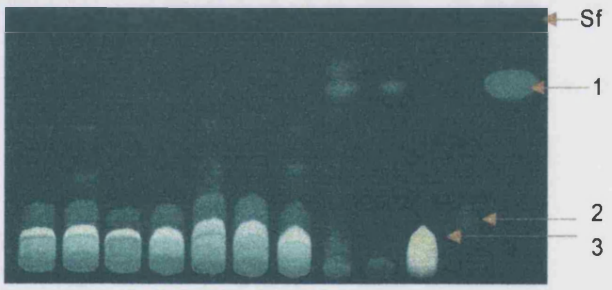



**Figure 5.10 Detection of coumarins and flavonoids in extracts of elicited cassava cells and leaves.**

HPTLC-separated cassava cell and leaf extracts stained with Neu's reagent and photographed under $366_{nm}$ light. **A**: SDW-treated control leaves at 0 h; **B**: SDW-treated control leaves at 0 h; **C**: Elicited leaves with yeast elicitor (50 µg/ml) at time course indicated; **D**: Elicited cells with

yeast elicitor (50 μg/ml) at 48 h; **E**: SDW-treated control cells at 48 h; **F**: Rutin reference; **G**: Kaempferol-3-*O*-rutinoside reference; **H**: Scopoletin reference; **Sf**: Solvent front (Sf= 13 cm); **1**: Scopoletin Rf = 0.59; **2**: Kaempferol Rf = 0.32; **3**: Rutin Rf = 0.21. Loading was 1 g of leaves in 50 μl of methanol and 10 g of cells in 50 μl of methanol, applied as a line of 1 cm to the HPTLC plate.

**Figure 5.10** shows that cassava leaves contained more secondary metabolites (unidentified compounds) than cassava cells. However, cassava cells contained more scopoletin than leaves, and a small increase in scopoletin in the elicited cells was apparent in comparison with control cells. Cassava leaves before and after elicitation contained flavonoids, including rutin and kaempferol-3-*O*-rutinoside. While rutin increased slightly in elicited leaves in comparison with the controls all these compounds were pre-existing in cassava cells rather than induced by the elicitor.

**Figure 5.11** examines the presence of scopoletin and scopolin in elicited cassava leaves. A peak of scopoletin was apparent in the elicited leaves after 48 h. It was interesting to observe detectable amounts of scopoletin in the control leaves by 96 h. The experimental protocol for these assays necessitated wounding the leaves and it is probable that the scopoletin in the controls was induced by this wounding, while the earlier scopoletin peak in the elicited leaves was due to the superimposition of elicitation on wounding. While it is less clear in the figure, similar observations can be made for scopolin.

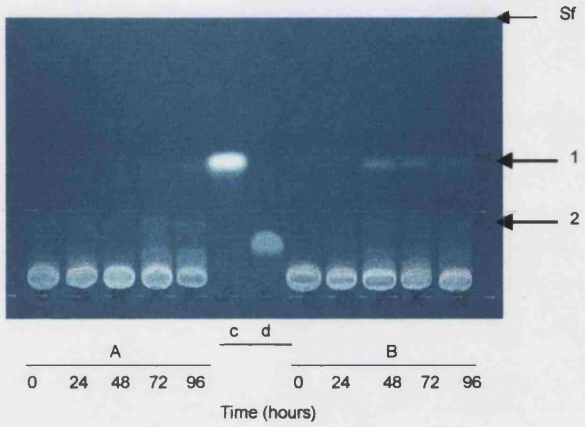



**Figure 5.11 Detection of coumarins from elicited cassava leaves.**

HPTLC-separated cassava leaf extracts stained with Neu reagent and photographed under $366_{nm}$ light. **A**: SDW-treated control leaves; **B**: Elicited leaves with yeast elicitor (50μg/ml); **c**: Scopoletin reference; **d**: Scopolin; **Sf**: Solvent front (Rf = 13 cm); **1**: Scopoletin in elicited leaves with Rf = 0.59; **2**: Scopolin band with Rf = 0.18. Loading was 1 g of leaves in 50 μl of methanol applied as a line of 1 cm to the HPTLC plate.

HPTLC-plates were informative for the preliminary analysis and identification of some compounds. However, for conclusive identification it was necessary to perform HPLC of the cassava extracts from cells and leaves and UV comparison with commercial or acquired references compounds. HPLC chromatograms of extracts from cassava cells, the medium in which they had been grown, and leaves are shown **(Figure 5.12)**. Confirmation of the principal compounds was achieved by comparison of UV spectra and retention time of standard references **(Figures 5.13-5.15)**. The spectra were largely coincident indicating the likely identity of these four phenolics. Conclusive identification would require mass spectrometry or NMR.

In the elicited cells there was an increase in the number and size of compounds peaks compared to the control cells **(Figure 5.12 A)**, indicating an increase of secondary metabolites accumulation after 48 h as a result of elicitation. Three of these compounds scopolin, rutin, and kaempferol-3-*O*-rutinoside could be identified with confidence in the chromatogram. A contrast was that the medium in which the cells had been grown did not contain detectable compounds in either elicited or control samples **(Figure 5.12 B)**. In cassava leaves there was a change in abundance of some compounds between elicited and control samples, but no noticeable change in the number of compounds **(Figure 5.12 C)**. Scopoletin, scopolin, rutin, and kaempferol-3-*O*-rutinoside were readily identifiable. However, a compound at 6.5 min which decreased on elicitation and one at 10.5 min which increased were not identifiable. Comparing cassava cells and leaves, a more complex response in terms of the number of compounds was observed in elicited cells, while there were increases and decreases of only a few particular compounds in elicited leaves.

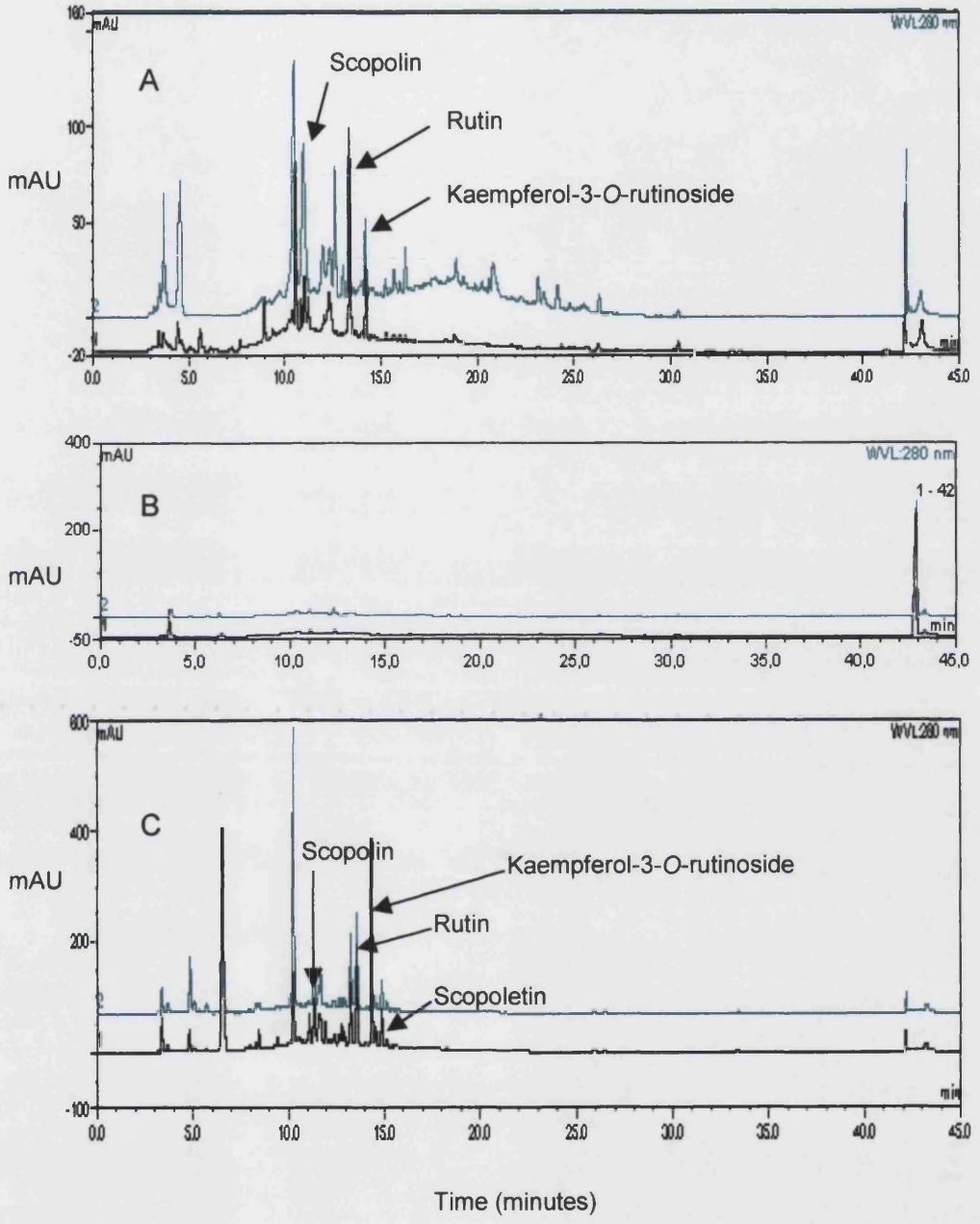



**Figure 5.12 HPLC detection of secondary metabolites in elicited cassava .**

**A**: Blue line - elicited cassava cells at 48 h; **Black line**: SDW treated control cassava cells; **B**: **Black line**: SDW-treated control medium at 48 h; **Blue line**: elicited medium at 48 h; **C**: **Blue line**: elicited cassava leaves; **Black line**: SDW-treated cassava leaves. 100μl of injection volume.

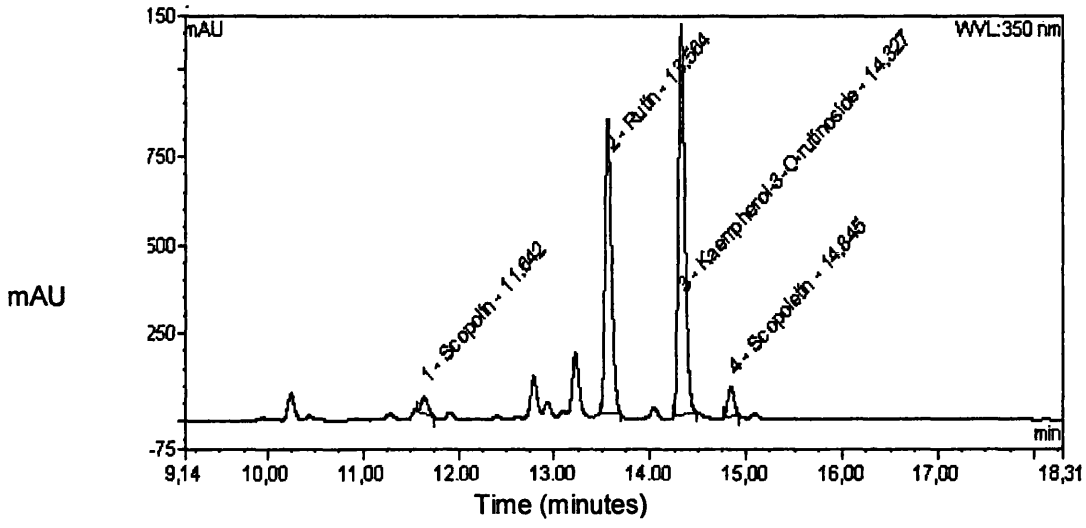



**Figure 5.13 Confirmation of identity of phenolic compounds by retention times in cassava cell extract.**

**1**: Scopolin (Rt = 11,642); **2**: Rutin (Rt = 13,564); **3**: Kaempferol-3-*O*-rutinoside (Rt = 14,327); **4**: Scopoletin (Rt = 14.845). The previous peaks were obtained from cassava cells extracts and analyses were made using HPLC software. Commercial references presented the following retention times: Scopolin (Rt = 10.72), rutin (Rt = 13,572), kaempferol-3-*O*-rutinoside (Rt = 13,782), and scopoletin (Rt = 14,737). Differences of retention times between standard references and cassava cell extracts can occur due to the purity of the standard reference in comparison with the cassava extract.

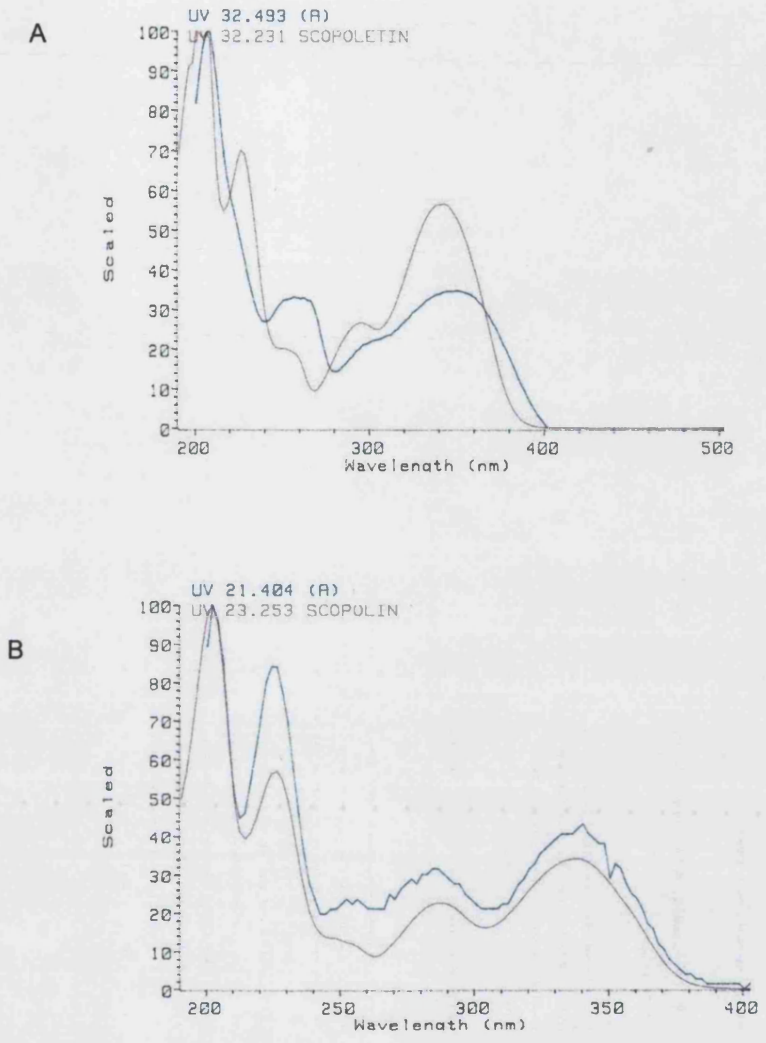
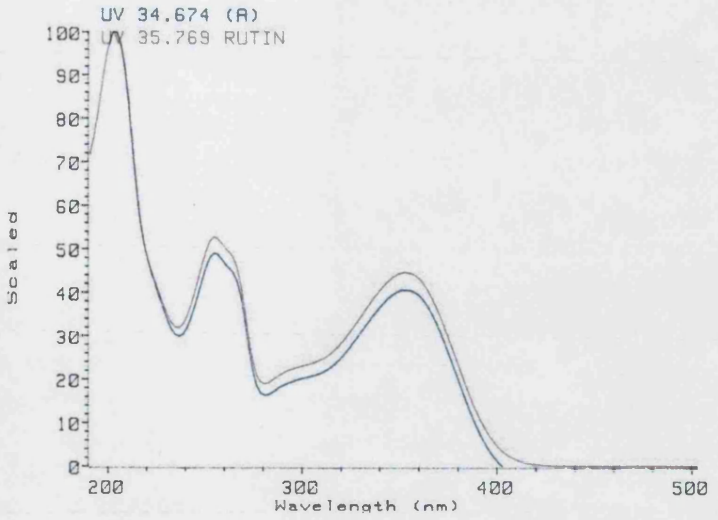



**Figure 5.14 UV confirmation of identity of phenolic compounds in cassava**

**A**: Scopolin; **Red line**: Standard reference; **Blue line**: cells extract; **B**: Scopoletin; **Red line**: Standard reference; **Blue line**: Cells extract

C




D

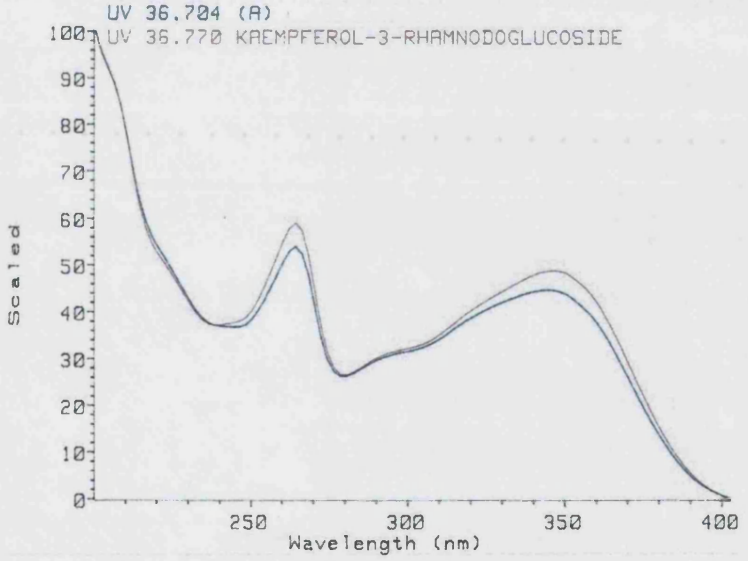



**Figure 5.15 UV confirmation of identity of phenolic compounds in cassava.**

**C**: Rutin; **Red line**: Standard reference; **Blue line**: Cells extract; **D**: Kaempferol-3-*O*-rutinoside; **Red line**: Standard reference; **Blue line**: Cells extract.

## 2.4 QUANTIFICATION OF PHENOLIC COMPOUNDS OF CASSAVA CELLS AND LEAVES

Phenolic compounds including coumarins and flavonoids were detected in cassava, some of which appeared to change in abundance in response to elicitation **(Figures 5.10, 5.11)**. Therefore, attempts were made to quantify changes during elicitation of cassava cells and leaves by HPLC and reference to known amounts of reference samples **(Figures 5.16-5.23)**. A summary of the quantification values is given in **Tables 5.1, 5.2**.

| Phenolic compound (nmol/g FW) | Control cells | Elicited cells |
|---|---|---|
| **Scopoletin** | 0.038-0.048 | 0.004-0.068 |
| **Scopolin** | 0.077-0.918 | 0.016-0.712 |
| **Rutin** | 0.333-2.119 | 0.116-1.287 |
| **Kaempferol*** | 0.448-1.543 | 0.093-1.009 |

**Table 5.1 Quantification of scopoletin, scopolin, rutin, and kaempferol-3-*O*-rutinoside in control and elicited cassava cells.**

* Kaempferol-3-*O*-rutinoside. The upper and lower extremes of amounts detected for each treatment are shown.

**Table 5.1** shows that kaempferol-3-*O*-rutinoside generally highest levels and scopoletin lowest. However, considerable variation, evident between control and elicited cells at time 0 h prevents any critical comparisons to be made.

| Phenolic compound (nmol/g FW) | Control leaves | Elicited leaves |
|---|---|---|
| **Scopoletin** | 0.253-2.584 | 1.153-4.21 |
| **Scopolin** | 0.890-4.032 | 2.236-6.003 |
| **Rutin** | 2.064-5.348 | 1.990-9.610 |
| **Kaempferol*** | 9.281-17.67 | 10.12-20.47 |

**Table 5.2 Quantification of scopoletin, scopolin, rutin, and kaempferol-3-*O*-rutinoside in control and elicited cassava leaves.**

* Values are average of three replicates. The upper and lower extremes of amounts detected for each treatment are shown.

From the values shown in **Table 5.2** it appeared that the overall quantities of these phenolic compounds in elicited leaves were higher than the control at time 0 h, which suggests that wounding the leaves had an effect in the leaves in addition of the elicitation process. Unfortunately, no independent replicates were measured (it was not practicable because of availability of the HPLC equipment). **Table 5.2** shows that kaempferol-3-O-rutinoside generally highest levels in elicited leaves.

**Figure 5.16** suggests that there may have been an early increase in scopolin during elicitation, though subsequently there was no consistent pattern.

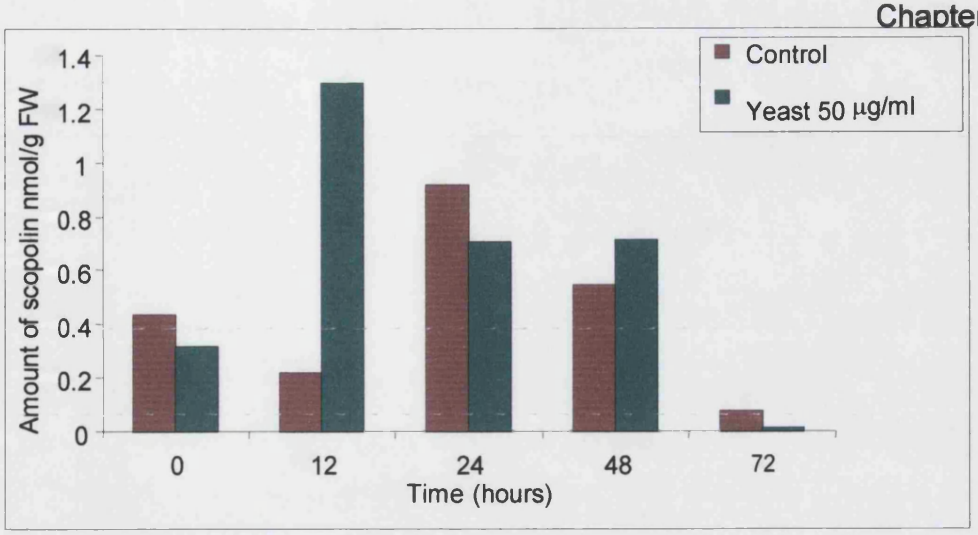



**Figure 5.16 Scopolin in cassava cells during elicitation.**

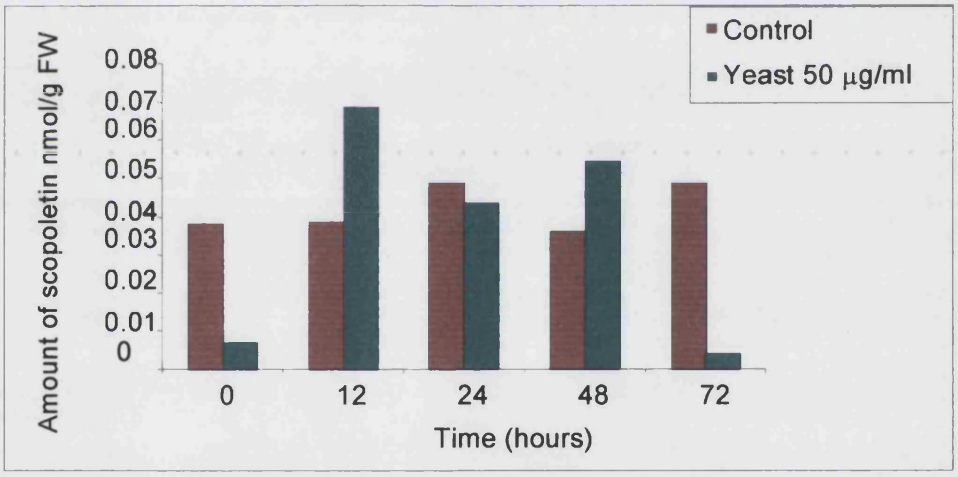



**Figure 5.17 Scopoletin in cassava cells during elicitation.**

The formation of scopoletin may occur from precursor molecules such as scopolin or esculetin or *via* ferulic acid has been proposed (Wheatley & Schwabe 1985). Scopoletin was present in all samples, but due to the lack of replicates it was not possible to detect any trend of accumulation in either the elicited or control

samples **(Figure 5.17)**. Certainly it was not possible to draw any conclusions as to the relationship between scopoletin or its possible precursor, scopolin.

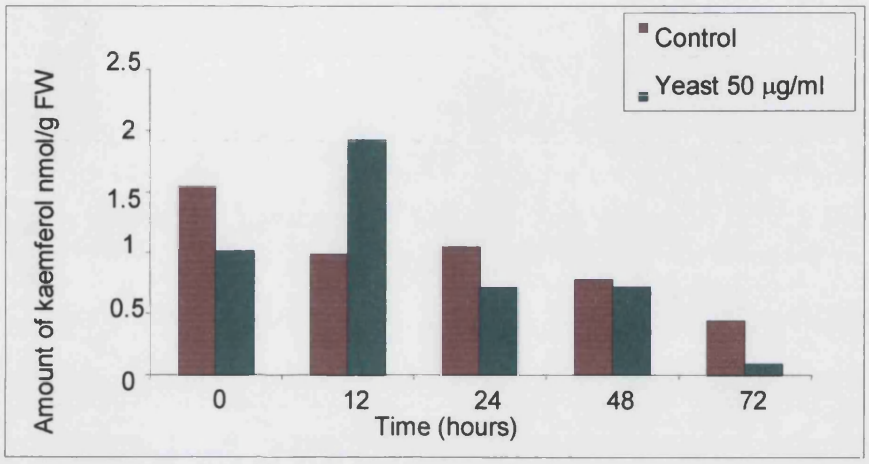



**Figure 5.18 Kaempferol-3-*O*-rutinoside in cassava cells during elicitation.**

While, for the same reasons, it is difficult to have full confidence in the measures of kaemferol-3-*O*-rutinoside, there appears to be a general decrease in amount of this compound with time in both elicited and control cells **(Figure 5.19)**.

Again with rutin, as with the preceding phenolics, while the method detected its presence it was not possible to measure with confidence differences in abundance with time or between elicited and control samples **(Figure 5.19)**.

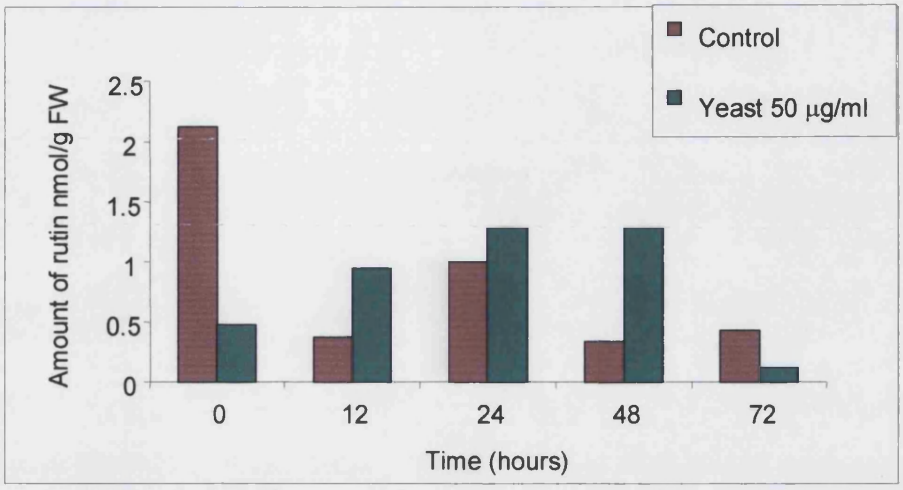



**Figure 5.19 Rutin in cassava cells during elicitation.**

Similar experiments were performed using elicited leaves in order to compare the response in the cells to that *in planta* **(Figures 5.20-5.23)**. However, in these cases three independent replicates could be used. However, in all cases there were high standard errors and it was difficult to detect trends with certainty. Scopolin, scopoletin and kaempferol-3-*O*-rutinoside appeared more abundant in the elicited leaves than the controls, while rutin was less abundant.

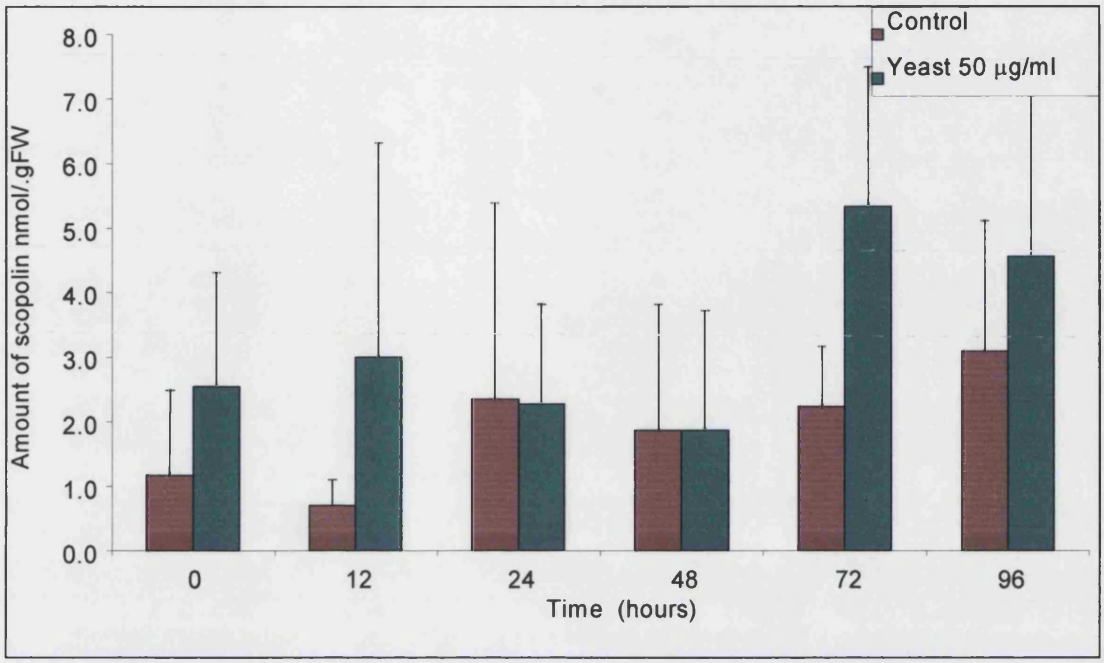



**Figure 5.20 Scopolin in cassava leaves during elicitation.**

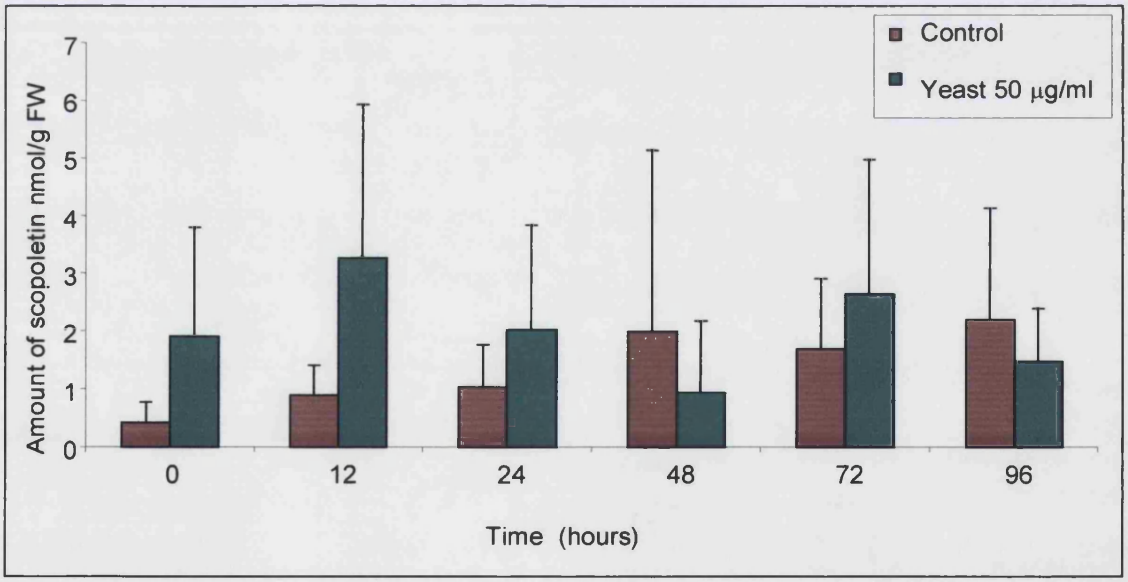



**Figure 5.21 Scopoletin in cassava leaves during elicitation**

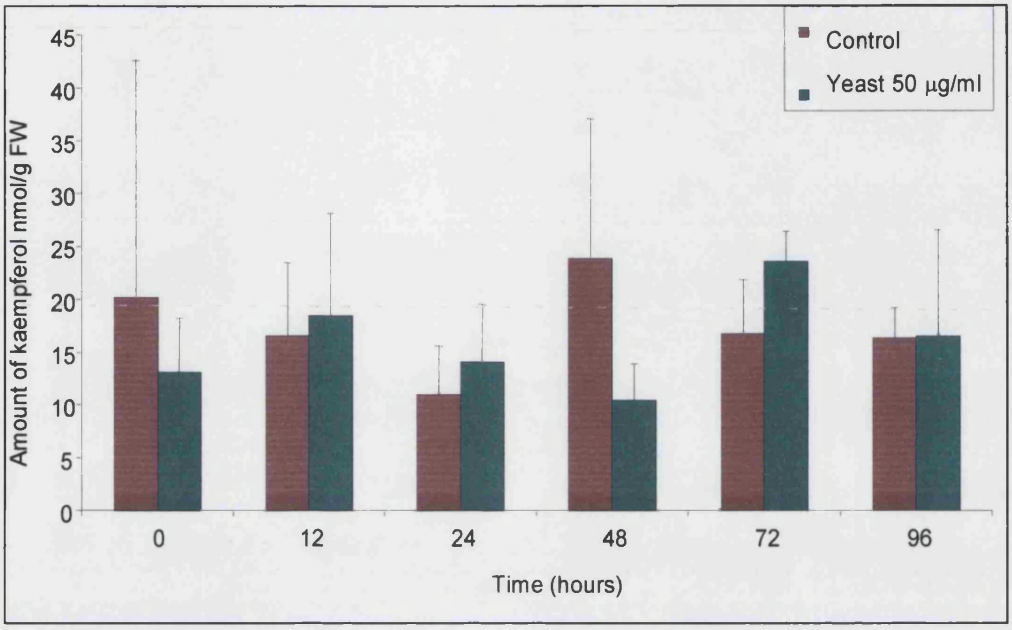



**Figure 5.22 Kaempferol-3- rutinoside in cassava leaves during elicitation.**

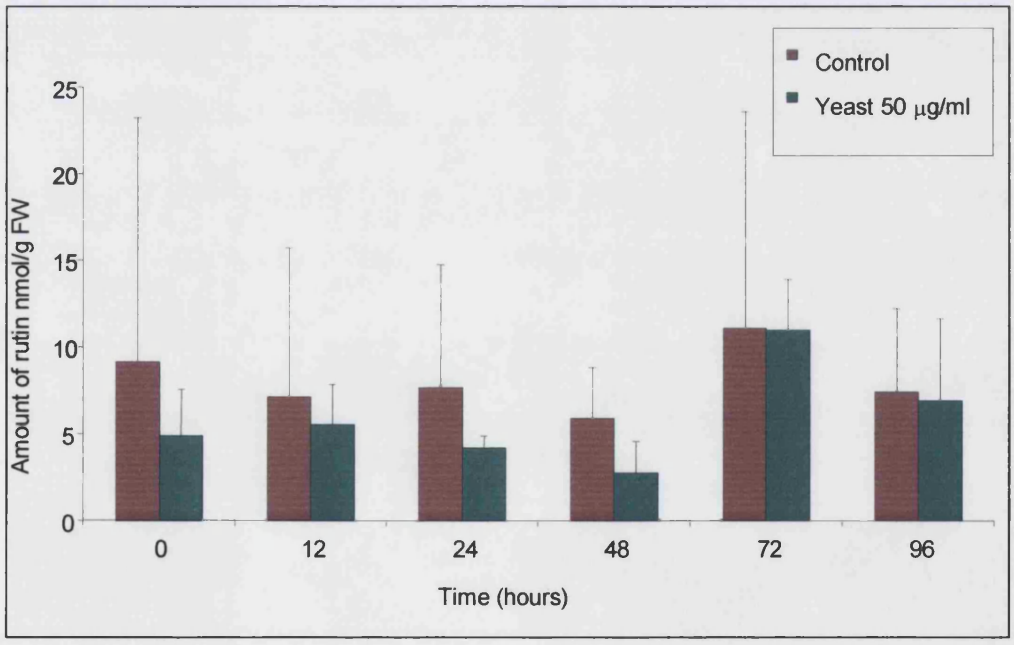



**Figure 5.23 Rutin in cassava leaves during elicitation.**

## 2.5 ANTIMICROBIAL ACTIVITY OF CASSAVA CELL EXTRACTS

### 2.5.1 TLC Bioassays with *T. harzianum*

Crude extracts from cassava cells were separated by TLC and the plates used in bioassays with *T. harzianum*, *F. avenae* and *Xam* (strain 2967) **(Figures 5.24-5.26)**.

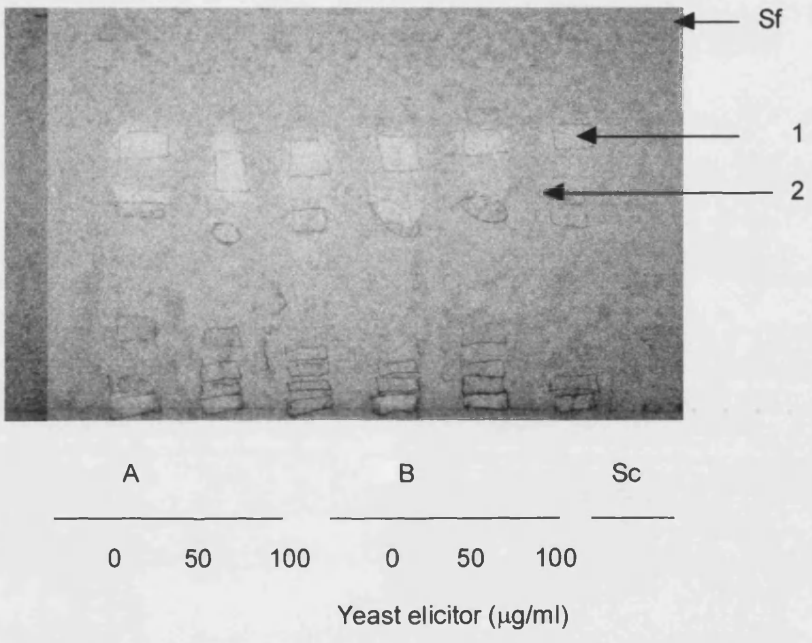



**Figure 5.24 TLC bioassay with cassava cell extracts and *T. harzianum*.**

Bioassay after 4 days at 26 °C in the dark. Samples of 48 h after elicitation were applied in line of 1 cm to the TLC plate. **A**: (**0**: SDW control treated cells; **50**: Elicited cells with yeast (50 μg/ml) extracted with pH 5.0; **100**: Elicited cells with yeast (100 μg/ml) extracted with pH 5.0); **B**: (**0**: SDW control treated cells; **50**: Elicited cells with yeast (50 μg/ml) extracted with pH 8.0; **100**: Elicited cells with yeast (100 μg/ml) extracted with pH 8.0). Inhibition zones are indicated; **1**: Inhibition zone with Rf= 0.69; **2**: Inhibition zone with Rf= 0.49. **Sf**: Solvent front; **Sc**: Solvent control. Rfs for all zones marked given in **Appendix 5**.

**Figure 5.24** shows a bioassay with *T. harzianum* with three inhibition zones (RFs: 0.0; 0.49, and 0.69), which did not correspond with the Rfs of the compounds identified before in the chromatograms such as scopoletin, rutin, kaempferol-3-*O*-rutinoside or scopolin. Rfs for these four phenolics on a TLC plate, which was run in parallel, were scopoletin (0.59), scopolin (0.18); Rutin (0.21), and kaempferol-3-*O*-rutinoside (0.32). These results confirm the presence of fungitoxic compounds in cassava cells, other than the four phenolics tested individually. These inhibition zones were present also in both the elicited and control extracts which confirms previous experiments that suggest that phytoalexins are not synthesized *de novo* in cassava cells after they have been challenged with cell wall glucan elicitor, but some constitutive antifungal compounds are present in the cells. Also, no apparent difference was observed in the samples extracted at different pH.

2.5.2 TLC Bioassays with *Fusarium avenae*

Another fungus was tested (*Fusarium avenae*) against cell extracts which revealed different inhibition zones to the ones observed above with *T. harzianum* **(Figure 5.25)**.

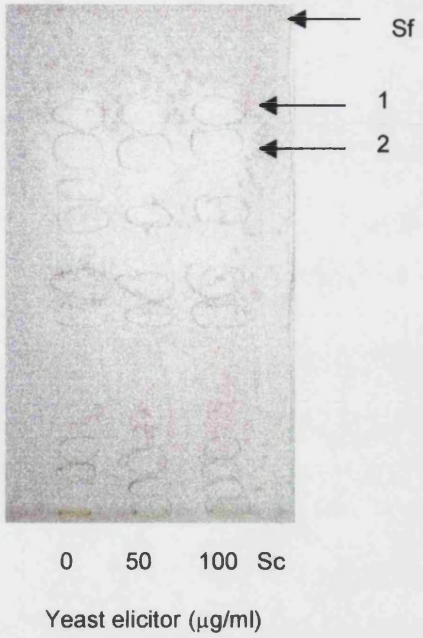



**Figure 5.25 TLC bioassay with cassava cell extracts and *F. avenae*.**

Bioassay after 4 days at 26 °C in the dark. Samples of 48 h after elicitation were applied in line of 1 cm to the TLC plate. **A**: SDW-treated control cells; **B**: Elicited cells with yeast (50 μg/ml). Inhibition zones are indicated; **1**: Inhibition zone with Rf= 0.89; **2**: Inhibition zone with Rf= 0.83. **Sf**: Solvent front; **Sc**: Solvent control. Rfs for all zones marked given in **Appendix 6**.

**Figure 5.25** shows inhibition zones again that did not correspond with the Rfs of the phenolic identified previously such as scopoletin, scopolin, rutin, and kaempferol-3-*O*-rutinoside. However, the less polar fungitoxic compounds always run at the end of the plate showing less polarity than the ones near the baseline was the compounds to show antifungal activities with all the fungi tested.

2.5.3 TLC Bioassay with *Xam*

A bioassay with bacteria (*Xam*) was also performed but did not reveal any inhibition zones in a range of concentration of cassava cell extracts **(Figure 5.26)**.

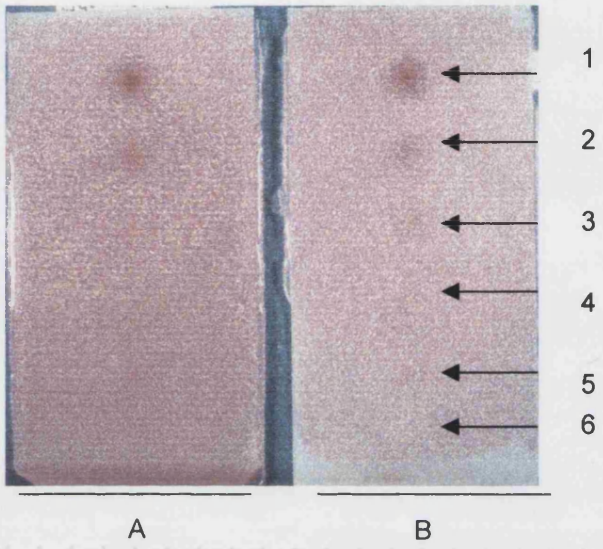



**Figure 5.26 TLC bioassay with *Xam* with range of concentration of cassava cells extracts.**

TLC bioassay after 3 days in a moisturized chamber at 26 °C at dark. Samples of 48 h after elicitation were applied and dried as spots in a TLC plate and an overlay layer with *Xam* at $10^8$ cfu. $ml^{-1}$ was applied over the TLC plate. **1**: 2 g of cassava elicited cells extract; **2**: 2.5 g of cassava elicited cells extract; **3**: 1.5 g of cassava elicited cells extract; **4**: 1.0 g; **5**: 0.5 g; **6**: Solvent control; **A**: SDW-treated control cells; **B**: Elicited cells with yeast (50 μg/ml).

**Figure 5.26** shows that no apparent effect of cassava cell extracts on bacterial growth even at the highest extract tested. Note the enhanced bacterial growth around cell extracts spots especially from elicited cells.

## 2.6 ANTIMICROBIAL ACTIVITY OF FOUR MAJOR CASSAVA PHENOLICS

Four phenolic compounds were identified in cassava cells and leaves. Notwithstanding that none of these phenolic compounds increased after elicitation they may still play a role in the antimicrobial defence of the plant. One of them, scopoletin, has been identified as an antimicrobial in another member of *Euphorbiaceae* family, rubber tree (*Hevea brasiliensis*) against *Corynespora cassiicola* (Breton et al. 1997). In cassava roots undergoing PPD, other phenolic compounds were also detected which increase in abundance, these included, ferulic acid, esculetin and quercetin (J.R.Beeching personal communication). Therefore, scopoletin, ferulic acid, esculetin and quercetin were tested for *in vitro* fungitoxicity. The effect of a range of concentrations of these phenolic compounds were assayed against spore germination, germ tube length, and mycelial growth of cassava phytopathogenic fungi, *Fusarium solani*, *Fusarium oxysporum,* and the saprotroph *Trichoderma harzianum.* Additionally, the fungitoxicity of these phenolic compounds after oxidation by peroxidase or tyrosinase was assessed. Finally the fungitoxicity and antibacterial effects of crude extracts from cassava cells and leaves were assayed.

### 2.6.1 Effect of Concentration of Four Phenolic Compounds on the Germination of *T. harzianum*, *F. solani*, and *F. oxysporum.*

Microscope slide bioassays were made in order to test the fungitoxicity of four major phenolic compounds found in cassava, esculetin, ferulic acid, quercetin, and scopoletin. **Table 5.3** summarizes the range of effective concentrations of

phenolic compounds required to reduce germination and germ tube length to 50% of the solvent (ethanol) control ($EC_{50}$). These summary data show that dependent on the phenolic and fungi tested widely different concentrations were required to inhibit germination and germ tube elongation, of the two phytopathogenic fungi *Fusarium solani*, and *Fusarium oxysporum*, and a sapotroph *Trichoderma harzianum* **(Figures 5.27-5.32)**.

| | ***T. harzianum*** | | ***F. solani*** | | ***F. oxysporum*** | |
|---|---|---|---|---|---|---|
| **Phenolic compound** | **Germination** | **Germ tube length** | **Germination** | **Germ tube length** | **Germination** | **Germ tube length** |
| **Esculetin** | 100-200 | > 1000 | > 1000 | 200-500 | > 1000 | 100-200 |
| **Ferulic acid** | 50-100 | 50-100 | 500-1000 | 100-200 | > 1000 | 50-100 |
| **Quercetin** | > 1000 | 50-100 | > 1000 | 100-200 | > 1000 | > 1000 |
| **Scopoletin** | 200-500 | 10-50 | > 1000 | 100-200 | > 1000 | 50-100 |

**Table 5.3 The range of effective concentrations (μg/ml) of phenolics required to reduce germination and germ tube length to 50% of the control ($EC_{50}$).**

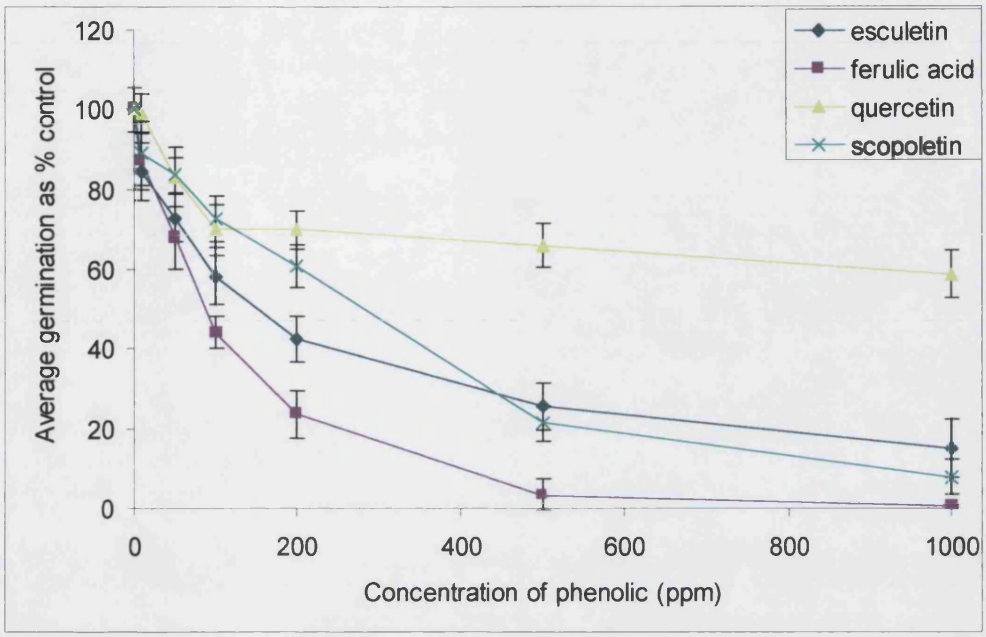



**Figure 5.27 Effect of the concentration of four phenolics on the germination of *T. harzianum*.**

Standard error of three independent experiments.

**Figure 5.27** represents effects of concentration of four phenolics upon germination of *T. harzianum*, which is a saprotroph rather than a pathogen of cassava. A range of concentration was tested for each phenolic compound (10, 50, 100, 200, 500, 1000 μg/ml). At 100 μg/ml, esculetin, ferulic acid, quercetin and scopoletin reduced germination to *ca*. 58%, 44%, 70% and 72.5% of the control respectively. These changes represent a significant decrease in percentage of germination. At the highest concentration 0f 1000 μg/ml all four phenolic compounds had reduced germination further to 15 %, 0.42 %, 59 %, and 7.8% control respectively. The error bars shown in Figure 5.25 suggest that changes in percentage germination between treatments were not always significant. ANOVA

analysis was used and showed that esculetin and ferulic acid inhibited germination significantly more with each increase in concentration (for details of these statistically test and subsequent ones in these chapter see **Appendix 7**). Quercetin, inhibited germination to approximately 65% of the control at all concentrations above 100 μg/ml, and scopoletin gave similar level of germination to the control at 50 and 10 μg/ml.

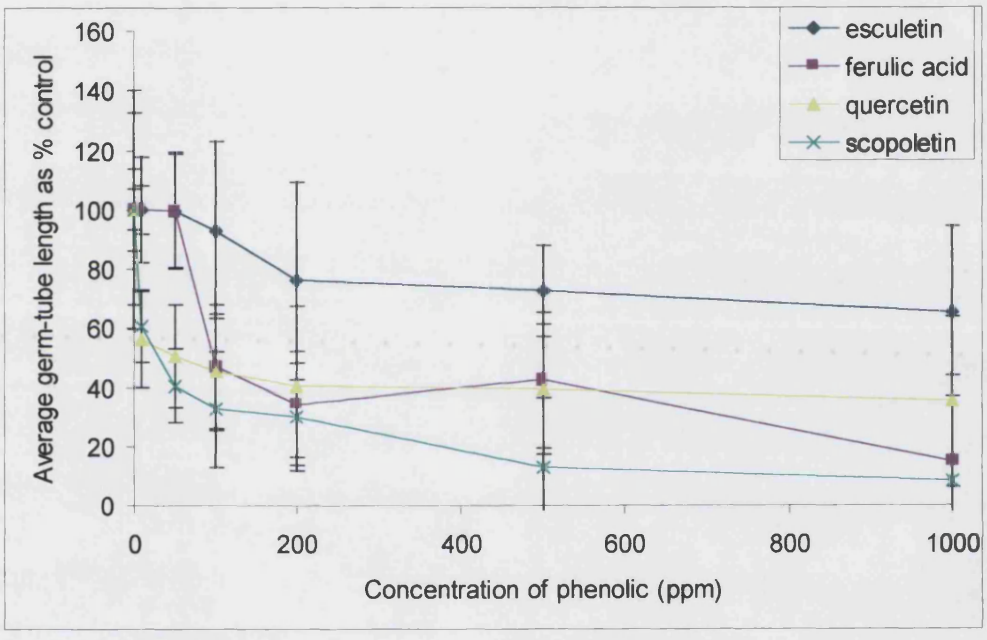



**Figure 5.28 Effect of the concentration of four phenolics on the germ tube length of *T. harzianum*.**

Standard error of three independent experiments.

Germ tube length tended to decrease as phenolic concentration increased, however ANOVA analysis showed that differences were not always significant. At 200 μg/ml esculentin, ferulic acid, quercetin and scopoletin gave germ tubes of

76%, 34%, 40%, and 30% of the control respectively. These significant changes in germ tube length did not necessarily correspond with the changes in germination. For example, quercetin had least effect on germination while it had a large effect on germ tube length, while esculetin, which had a substantial effect on germination, was least effective on germ tube length **(Figures 5.27, 5.28)**. On the other hand, scopoletin had an equally strong effect on both germination and germ tube length.

Figure 5.27 show that all phenolic compounds reduced average germination of *F. solani* conidia between 0 and 1000 μg/ml. The ANOVA test showed that these reductions were significant. However, the $EC_{50}$ **(Table 5.1)** are very high for all four phenolics, with only ferulic acid reducing germination to 50% of the control at a concentration of between 500 and 1000 μg/ml. As with *T. harzianum*, the most effective phenolic was ferulic acid followed by esculetin.

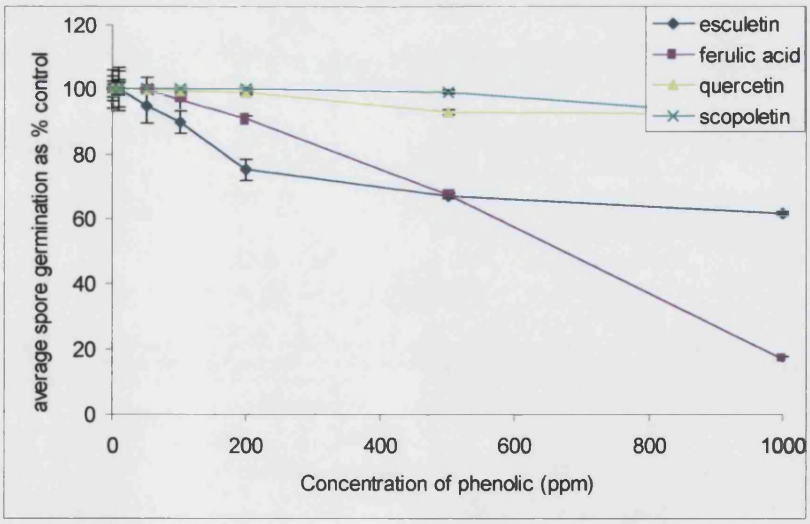



**Figure 5.29 Effect of the concentration of four phenolics on the germination of *F. solani*.**

Germination and germ tube elongation of *F. solani* were both affected by the four phenolic compounds tested **(Figures 5.29, 5.30)**. Germination of *F. solani* did not respond so dramatically to increases in concentration as did *T. harzianum*, in particular *F. solani* responded minimally to scopoletin and quercetin. On the other hand, inhibition of germ tube elongation in *F. solani* responded approximately uniformly to all four phenolics, in contrast to *T. harzianum*, which responded least to quercetin.

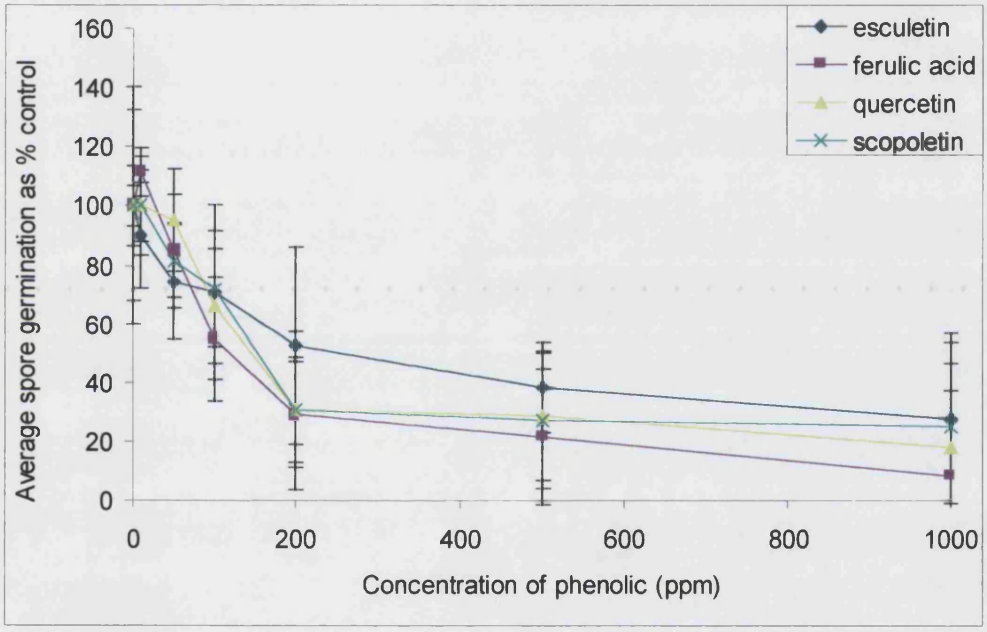



**Figure 5.30 Effect of the concentration of four phenolics on the germ tube length of *F. solani*.**

Finally a fungitoxicity test was made with *F. oxysporum*, a phytopathogenic fungus of cassava **(Figures 5.31, 5.32)**. Table 5.1 shows that none of the phenolic compounds gave an $EC_{50}$ below 1000 μg/ml, although ANOVA tests showed that

all compounds except for scopoletin give a significant drop in germination with increase in concentration. At 1000 μg/ml, ferulic acid reduced percentage germination to 60%; a lower level than any of the other compounds. However, this was not statistically significant, as ANOVA tests showed that esculetin, quercetin and ferulic acid were all equally fungitoxic throughout. Scopoletin did not have any significant effect on germination.

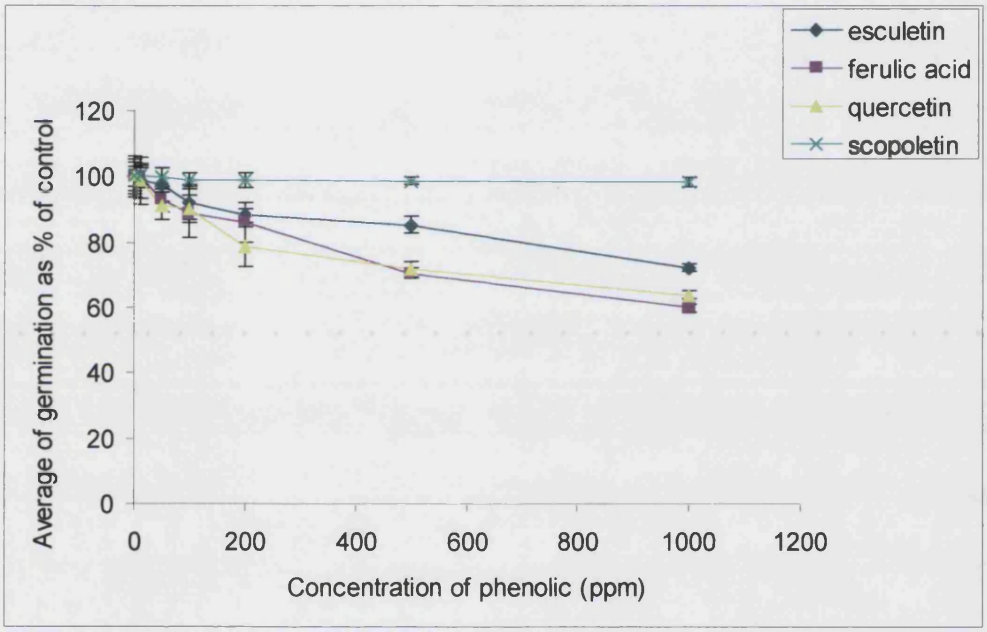



**Figure 5.31 Effect of the concentration of four phenolics on the germination of *F. oxysporum*.**

Germ tube length was more sensitive to the phenolics than germination in *F. oxysporum*. Scopoletin and ferulic acid had $EC_{50}$s of between 50 and 100 μg/ml, showing that, unlike its effects on percentage germination, scopoletin significantly reduced germ tube length. Esculetin was less effective than scopoletin or ferulic

acid in reducing germ tube length, giving an $EC_{50}$ of between 100 and 200 μg/ml. Quercetin was the least effective, giving an $EC_{50}$ of over 1000 μg/ml. Although Figure 5.30 shows that ferulic acid and esculetin had a marked effect on germ tube length, an ANOVA test revealed that neither had had any statistically significant effect on germ tube length compared with the control, although the means did tend to drop.

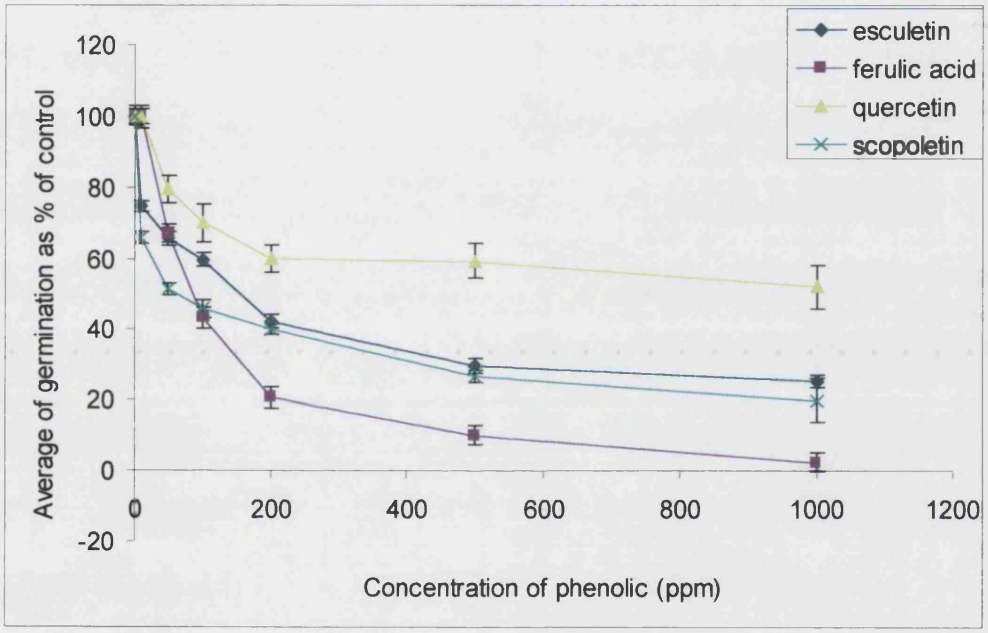



**Figure 5.32 Effect of the concentration of four phenolics on the germ tube length of *F. oxysporum*.**

Examining the overall results of the phenolics on these three fungi, not all of which were significant statistically, it was apparent that ferulic acid had the most obvious antifungal effects. However, though a compound applied to a fungal colony may lead to reduced fungal growth relative to that of an untreated one this does not necessarily imply direct antifungal activity of the compound *per se*.

Rather, it might reflect non-specific alterations in the physiological environment (e.g. pH, osmotic pressure), with consequent adverse effects on growth. Ferulic acid could have an antimicrobial effect at high concentrations purely due to pH effects. Therefore, the pH of the ferulic acid solutions at different concentrations was determined and proved to range from pH 5.7 to 7.3 **(Table 5.4)**.

| Concentration of ferulic acid (μg/ml) | pH |
|---|---|
| 1000 | 5.7 |
| 500 | 5.9 |
| 200 | 6.2 |
| 100 | 6.6 |
| 50 | 7.0 |
| 10 | 7.3 |

**Table 5.4 pH against concentration of ferulic acid**

All fungi tested on a microslide bioassay were capable of normal germination and germ tube elongation in Czapek Dox solution at pH 5.5-7.5 which rules out the possible fungitoxic effects of pH when testing ferulic acid.

2.6.2 Effect of Four Phenolic Compounds on the Mycelial Growth of *T. harzianum*, *F. solani*, and *F. oxysporum*

The four phenolic compounds tested above showed varying degrees of inhibition of germination and germ tube elongation of three fungal species, though none of them completely inhibited germination at the concentrations tested. In order to determine the effects of these compounds on the further development of the fungi experiments were carried out on their inhibition of fungal mycelial growth.

A range of concentrations of these phenolic compounds (210,50,200,500, and 1000 μg/ml) were applied to paper discs and placed on plates on which mycelial of the fungi had been grown for 48 h. After 48 h of incubation in the dark, the expected inhibition zones around the paper discs were not observed with any of the phenolic compounds in fungal combinations. Even at the highest concentrations of these compounds no inhibition zone was observed. *F. solani* and *T. harzianum* in particular had even colonized areas immediately adjacent to the paper discs in preference to the control discs **(Figure 5.33)**. The sapotroph fungi *T. harzianum*, after a further 48 h incubation, had also begun to colonize both the control and the phenolic-treated paper discs **(Figure 5.34)**. These results show that while the four phenolic compounds were capable of inhibition germination they had no visible effect on mycelial growth at even the highest concentrations tested. Therefore, these compounds could play a role in the prevention of establishment of these fungi but once they have germinated the phenolics would not able to prevent the spread of the fungi.

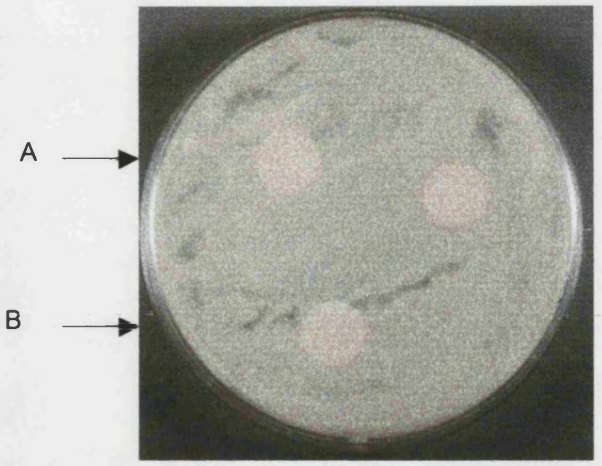



**Figure 5.33 Mycelial growth of *F. solani* with scopoletin.**

**A**: SDW treated control paper disc; **B**: Scopoletin (1000 μg/ml) paper disc. 48 h of incubation in the dark and at 25°C.

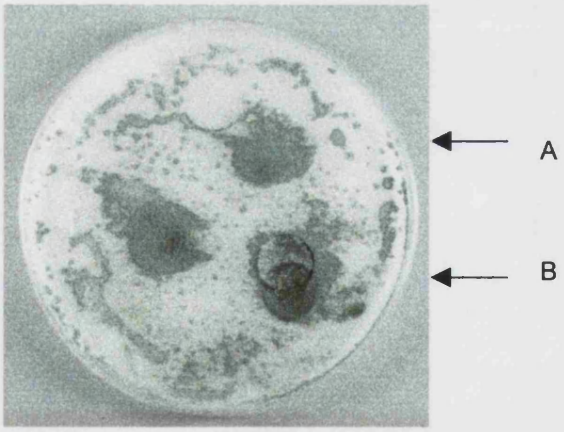



**Figure 5.34 Mycelial growth of *T. harzianum* with quercetin**

**A**: Quercetin (1000 μg/ml) paper disc; **B**: SDW treated control paper disc. 96 h of incubation in the dark and at 25°C.

2.6.3 Effect of Oxidation on Phenolic Toxicity

The phenolic compounds had been shown to have limited fungitoxicity which depended on the growth state of the fungi. *In vivo*, of course, these compounds are not present in isolation, in particular there are enzymes present that can modify them, perhaps to a more toxic form. For example, peroxidase was shown in this study to increase in cassava cells and leaves after elicitation (Chapter 4). Peroxidase and tyrosinase oxidise phenolic compounds, which may be involved in or simply reflect resistance, as activities are higher in compatible than in incompatible reactions (Valle et al. 1997). Scopoletin toxicity has been shown to be enhanced *in situ* by peroxidase (Valle et al. 1997). Therefore, a preliminary test was made to ascertain the oxidation of scopoletin, rutin, esculetin, ferulic acid, and quercetin by peroxidase and tyrosinase **(Figures 5.35, 5.36)**.

Peroxidase oxidized esculetin and scopoletin even at low levels of hydrogen peroxide as was observed in the microtitre plates by esculetin changing from transparent to bright yellow and scopoletin turning blue **(Figure 5.35)**. Esculetin was the only phenolic compound, which was oxidized by tyrosinase and was detected by a change from transparent to a yellow colouration **(Figure 5.36)**. Therefore, fungitoxic effects of scopoletin and esculetin oxidized by peroxidase and esculetin oxidized by tyrosinase was determined.

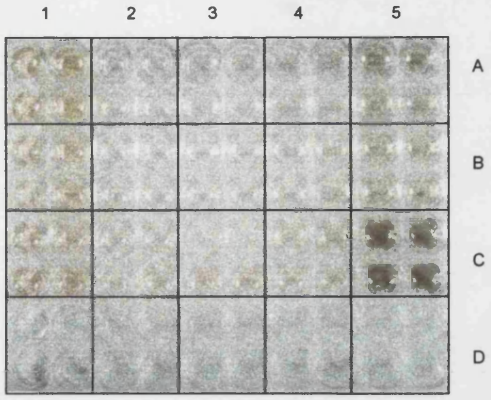



**Figure 5.35 Oxidation of esculetin and scopoletin by peroxidase.**

**1**: Esculetin; **2**: Ferulic acid; **3**: Quercetin; **4**: Rutin; **5**: Scopoletin. 200 μl of phenolic compounds were prepared y dissolving 1000 μg/ml of the phenolic compound in 0.5 M phosphate buffer pH 6.0 and making to 10 ml with SDW finally, 250 μl was added to the microtitre plate and mixed with hydrogen peroxide and peroxidase. **A**: 80 μl $H_2O_2$ (1 %); **B**: 200 μl $H_2O_2$ (1 %); **C**: 400 μl $H_2O_2$ (1 %); **D**: Control made with all the reagents except phenolic compound.

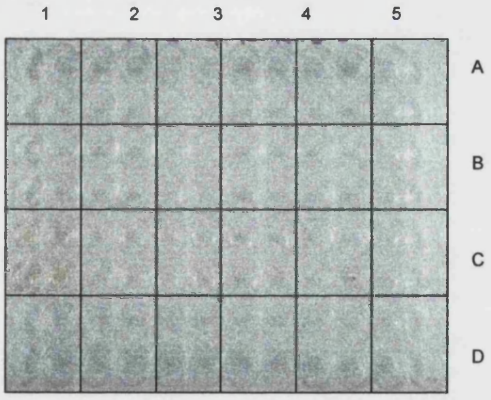



**Figure 5.36 Oxidation of esculetin by tyrosinase.**

**1**: Esculetin; **2**: Ferulic acid; **3**: Quercetin; **4**: Rutin; **5**: Scopoletin. 200 μl of phenolic compounds were prepared y dissolving 1000 μg/ml of phenolic compound into 0.5 M phosphate buffer pH 6.0 and making to 10 ml with SDW finally 250 μl was added to the microtitre plate and mixed with

hydrogen peroxide and 20µl of peroxidase. **A**: 80 µl $H_2O_2$ (1 %); **B**: 200 µl $H_2O_2$ (1 %); **C**: 400 µl $H_2O_2$ (1 %); **D**: Control (20 µl of peroxidase II mixed with 0.5 M phosphate buffer pH 6.0 up to 250 µl).

### 2.6.4 Fungitoxic Effects of Oxidized Phenolics on the Germination and Germ Tube Length of *T. harzianum*, *F. solani and F. oxysporum*.

A microslide bioassay was performed with oxidized esculetin and scopoletin in order to determine their toxicity towards fungal germination and germ tube length. Different amounts of hydrogen peroxide and peroxidase were used as the levels available *in planta* were unknown and this was a preliminary experiment to evaluate the potential toxicity of the quinone moiety. **Table 5.5** shows the response of *T. harzianum*.

| Treatment number | Added to spore suspension | Average spore germination (% control) | Average germ tube length (% control) |
|---|---|---|---|
| 1 | Esculetin + peroxidase + 0.1 % $H_2O_2$ | 0.00 | 0.00 |
| 2 | Esculetin + peroxidase + 0.05 % $H_2O_2$ | 53.00 | 21.79 |
| 3 | Esculetin + peroxidase + 0.02 % $H_2O_2$ | 71.00 | 43.08 |
| 4 | Esculetin + tyrosinase | 28.00 | 19.49 |
| 5 | Scopoletin + peroxidase + 0.1 % $H_2O_2$ | 26.00 | 20.77 |
| 6 | Scopoletin + peroxidase + 0.05 % $H_2O_2$ | 40.00 | 42.31 |
| 7 | Scopoletin + peroxidase + 0.02 % $H_2O_2$ | 70.00 | 44.62 |
| 8 | Buffer + peroxidase + 0.1 % $H_2O_2$ | 100.00 | 98.21 |
| 9 | Buffer + peroxidase + 0.05 % $H_2O_2$ | 100.00 | 99.74 |
| 10 | Buffer + peroxidase + 0.02 % $H_2O_2$ | 100.00 | 100.00 |
| 11 | Buffer + peroxidase | 100.00 | 100.00 |
| 12 | Buffer + tyrosinase | 100.00 | 100.00 |
| 13 | Buffer only | 100.00 | 100.00 |

**Table 5.5 Effect of oxidized phenolics on the germination and germ tube length of *T. harzianum*.**

Treatments 8-13 (treatments containing no phenolic compound) **Table 5.5** did not differ significantly (ANOVA) in average percentage germination or average germ tube length and were *ca.* 100% of the control (treatment 13). This proves only the presence of the phenolic compound could be affecting the growth of the pathogen. Oxidised scopoletin affected germ tube growth more than it affected germination and reduced germ tube length to less than 50 % of that of the control at all levels of available hydrogen peroxide. Esculetin with tyrosinase reduced both average germination and germ tube length to less than 25 % of that of the control.

Scopoletin and esculetin with peroxidase had a greater effect on germ tube length than on germination.

A similar experiment to that described above was performed with *F. solani* **(Table 5.6)**. Again, all treatments lacking phenolic compounds were not significantly (ANOVA) different from the control treatment in either germination or germ tube length. Germ tube length seemed to be more affected by the presence of phenolic compound than was germination. Unlike *T. harzianum*, none of the treatments reduce germ tube length below 45 % of the control and germination was not reduced by more than 52 % by any of the phenolic treatments. Higher concentrations of hydrogen peroxide tended to improve the fungitoxicity of both esculetin and scopoletin.

| Treatment number | Added to spore suspension | Average spore germination (% control) | Average germ tube length (% control) |
|---|---|---|---|
| 1 | Esculetin + peroxidase + 0.1 % $H_2O_2$ | 58.00 | 81.97 |
| 2 | Esculetin + peroxidase + 0.05 % $H_2O_2$ | 62.00 | 80.05 |
| 3 | Esculetin + peroxidase + 0.02 % $H_2O_2$ | 100.00 | 87.98 |
| 4 | Esculetin + tyrosinase | 72.00 | 77.32 |
| 5 | Scopoletin + peroxidase + 0.1 % $H_2O_2$ | 57.00 | 45.63 |
| 6 | Scopoletin + peroxidase + 0.05 % $H_2O_2$ | 91.00 | 64.75 |
| 7 | Scopoletin + peroxidase + 0.02 % $H_2O_2$ | 92.00 | 82.24 |
| 8 | Buffer + peroxidase + 0.1 % $H_2O_2$ | 100.00 | 98.63 |
| 9 | Buffer + peroxidase + 0.05 % $H_2O_2$ | 100.00 | 100.00 |
| 10 | Buffer + peroxidase + 0.02 % $H_2O_2$ | 100.00 | 98.36 |
| 11 | Buffer + peroxidase | 100.00 | 100.00 |
| 12 | Buffer + tyrosinase | 100.00 | 98.36 |
| 13 | Buffer only | 100.00 | 100.00 |

**Table 5.6 Effect of oxidized phenolics on the germination and germ tube length of *F. solani*.**

In contrast to the previous fungi, *F. oxysporum* germination was more markedly affected by the oxidized phenolics than was germ tube length **(Table 5.7)**. Germ tube growth in all, but treatments 1, 2, and 7 were not statistically different (ANOVA) from the control, whilst all treatments containing a phenolic compound were also not significantly different from one another (although these tended to give shorter germ tubes than the control treatments). As before, germination in treatments not containing a phenolic compound were not statistically

different from the control whilst those containing either scopoletin or esculetin were similar to one another but much lower than the control.

| Treatment number | Added to spore suspension | Average spore germination (% control) | Average germ tube length (% control) |
|---|---|---|---|
| 1 | Esculetin + peroxidase + 0.1 % $H_2O_2$ | 56.00 | 81.71 |
| 2 | Esculetin + peroxidase + 0.05 % $H_2O_2$ | 55.00 | 85.09 |
| 3 | Esculetin + peroxidase + 0.02 % $H_2O_2$ | 66.00 | 93.64 |
| 4 | Esculetin + tyrosinase | 74.00 | 80.52 |
| 5 | Scopoletin + peroxidase + 0.1 % $H_2O_2$ | 61.00 | 91.65 |
| 6 | Scopoletin + peroxidase + 0.05 % $H_2O_2$ | 77.00 | 92.05 |
| 7 | Scopoletin + peroxidase + 0.02 % $H_2O_2$ | 71.00 | 94.43 |
| 8 | Buffer + peroxidase + 0.1 % $H_2O_2$ | 100.00 | 99.40 |
| 9 | Buffer + peroxidase + 0.05 % $H_2O_2$ | 100.00 | 99.20 |
| 10 | Buffer + peroxidase + 0.02 % $H_2O_2$ | 100.00 | 99.80 |
| 11 | Buffer + peroxidase | 100.00 | 99.60 |
| 12 | Buffer + tyrosinase | 100.00 | 100.00 |
| 13 | Buffer only | 100.00 | 100.00 |

**Table 5.7 Effect of oxidized phenolics on the germination and germ tube length of *F. oxysporum*.**

These results show that with all the fungi tested oxidized esculetin and scopoletin had significantly enhanced fungitoxicity compared to the unoxidised forms. *T. harzianum* was particularly sensitive to the oxidized phenolics.

2.6.5 Fungitoxic Properties of Crude Plant Extracts on *F. solani.*

The increased toxicity of scopoletin and esculetin when oxidized implies that these phenolic compounds may well have antifungal properties when acting in concert with other factors *in vivo.* Therefore, the fungitoxicity of cell extracts was tested by the microslide bioassay on *F. solani* and *T. harzianum.*

The following tables show that both the elicited and unelicited extracts were toxic to both fungi which is illustrated by the complete inhibition of spore germination with extracts equivalent to 5 g/ml from elicited and unelicited suspension cells taken between 0 and 24 h **(Tables 5.8, 5.9)**. However, no significant effect was observed on germination or germ tube length when concentration was 0.05 g/ ml extract. As before, germ tube length tended to be more affected than germination. Elicited samples extracted between 0 and 72 h inhibited germ tube elongation significantly (ANOVA) more than unelicited extracts. Additionally, elicited samples extracted between 24 and 72 h inhibited germination significantly more than unelicited samples.

| Time extracted (hours) | Concentration of crude extract (g/ml) | Non-Elicited (germination % control) | Elicited (germination % control) | Non-elicited (germ tube % control) | Elicited germ (tube length % control) |
|---|---|---|---|---|---|
| 0 | 0.05 | 100 | 100 | 100 | 100 |
| 0 | 0.5 | 17.25 | 19.15 | 97.86 | 54.76 |
| 0 | 5 | 0.00 | 0.00 | 0.00 | 0.00 |
| 12 | 0.05 | 99.33 | 100 | 93.81 | 99.05 |
| 12 | 0.5 | 28.98 | 31.16 | 59.05 | 44.76 |
| 12 | 5 | 0.00 | 0.00 | 0.00 | 0.00 |
| 24 | 0.05 | 100 | 99.83 | 97.38 | 100 |
| 24 | 0.5 | 77.39 | 34.67 | 77.86 | 18.57 |
| 24 | 5 | 0.00 | 0.00 | 0.00 | 0.00 |
| 48 | 0.05 | 99.66 | 100 | 100 | 96.43 |
| 48 | 0.5 | 95.64 | 48.41 | 91.90 | 7.52 |
| 48 | 5 | 49.58 | 0.00 | 50.48 | 0.00 |
| 72 | 0.05 | 99.83 | 100 | 96.90 | 100 |
| 72 | 0.5 | 80.57 | 50.92 | 25.71 | 85.71 |
| 72 | 5 | 20.44 | 0.00 | 10.52 | 0.00 |
| Solvent control | 0 | 100 | 100 | 100 | 100 |

**Table 5.8 Fungitoxic properties of cassava cell extracts on *F. solani*.**

| Time extracted (hours) | Concentration of crude extract (g/ml) | Non-Elicited (germination % control) | Elicited (germination % control) | Non-elicited (germ tube % control) | Elicited (germ tube length % control) |
|---|---|---|---|---|---|
| 0 | 0.05 | 99.16 | 99.33 | 100 | 99.84 |
| 0 | 0.5 | 19.26 | 18.81 | 50.42 | 36.40 |
| 0 | 5 | 0.00 | 0.00 | 0.00 | 0.00 |
| 12 | 0.05 | 98.66 | 100 | 98.75 | 99.17 |
| 12 | 0.5 | 55.61 | 58.46 | 78.83 | 28.75 |
| 12 | 5 | 0.00 | 0.00 | 0.00 | 0.00 |
| 24 | 0.05 | 98.61 | 100 | 100 | 98.75 |
| 24 | 0.5 | 71.86 | 49.25 | 68.33 | 47.92 |
| 24 | 5 | 0.00 | 0.00 | 0.00 | 0.00 |
| 48 | 0.05 | 100 | 100 | 99.58 | 95.42 |
| 48 | 0.5 | 83.92 | 50.59 | 35.00 | 37.50 |
| 48 | 5 | 55.61 | 0.00 | 13.75 | 0.00 |
| 72 | 0.05 | 100 | 100 | 99.17 | 99.58 |
| 72 | 0.5 | 55.18 | 28.74 | 45.81 | 29.58 |
| 72 | 5 | 0.00 | 0.00 | 0.00 | 0.00 |
| Solvent control | 0 | 100 | 100 | 100 | 100 |

**Table 5.9 Fungitoxic properties of cassava cell extracts on *T. harzianum*.**

These data imply that a fungitoxic compound(s) is present in extracts from cassava suspension cells which contrast with the bioassays using discs individually impregnated with the four pure phenolic compounds. This suggest that either there were other fungitoxic compounds present in the cassava cells or that there was a synergistic effect between them.

## 3 DISCUSSION

Phenylpropanoid metabolism in cassava suspension cells and leaves was studied after challenge with glucan cell wall elicitor from baker's yeast. PAL activity profiles and the accumulation of phenolic compounds evident by browning during elicitation indicated that general phenylpropanoid metabolism was active during elicitation of cassava suspension cells. Browning events in suspension cells challenged with elicitors have been associated with cell death or decreased growth rate in other plant species (Davis & Ausubel 1989; Hahlbrock et al. 1981). However, decrease in growth rate did not occur in cassava suspension cells challenged with yeast elicitor **(Figure 5.2)**. Such changes in coloration of the cells have been attributed to the induction of PAL (Dornenburg & Knorr 1997; García-Pérez et al. 1998). Specifically, discoloration in parsley cells was due to the concomitant accumulation of coumarin derivatives in cells treated with soluble cell-wall fragments from three different phytopathogenic *Phytophthora* spp. (Kombrink & Hahlbrock 1986).

The hypersensitive response (HR) is a powerful defence mechanism used by plants against pathogen attack. It is characterized phenotypically by cell death at the site of the pathogen penetration (Graham & Graham 1999). While HR is a specific response of plants to interactions with incompatible pathogens, some of the later components of that response can be observed in plant cell-elicitor interactions. In the majority of the plant/elicitor systems browning of the cells has been associated with cell death, with an increase in PAL, peroxidase and polyphenol oxidase enzyme activities (García-Pérez et al. 1998). In cultured tobacco cells, oxidative burst and PAL induction were neither necessary nor sufficient for hypersensitive cell death or for scopoletin accumulation (Dorey et al. 1999). A set of defence responses (PAL

induction, oxidation burst, and phytoalexin formation) were reported in parsley cells with a fungal oligopeptide elicitor but HR was not triggered (Parker et al. 1991).

PAL is encoded by a small multi-gene family in most plants, usually of about four members, although potato PAL has been shown to have over 40 members and loblolly pine appears to have only a single gene (Lois & Hahlbrock 1992; Joos & Hahlbrock 1992; Whetten et al. 1998). Southern blots of cassava genomic DNA and sequencing of cassava PAL genomic and cDNA clones suggested that cassava PAL contains at least three family members (H Li and JR Beeching, unpublished results). PAL promoter activity in cassava showed both developmental and stress-regulated expression (H Li and JR Beeching, unpublished results). In other plants, PAL genes have been shown to be regulated developmentally, to be induced by wounding, and by pathogen attack (Collinge & Slusarenko 1987; Graymitsumune et al. 1999).

Marked increases in PAL synthesis and mRNA accumulation have been observed in response to elicitor treatment in a number of plant/elicitor systems (Edwards et al. 1985; Tepper et al. 1989). PAL is highly regulated during development and in response to a range of environmental factors including elicitation, wounding, dilution or "transfer effect" and light (Hahlbrock et al. 1981; Hotter 1997; Rickard 1982; Schröder et al. 1976). The "transfer effect" is an induction of PAL due to subculture dilution, which affects the cells for *ca.* 6-24 h after their transfer (Hahlbrock 1976; Ozeki & Takeda 1994). In the experiments described in this chapter any transfer effect was avoided by using suspension cells that has been in subculture for five days. A transient increase in enzyme activity was preceded by increased PAL gene expression after elicitation in cassava in a dose-dependent manner, similar to results from other plant/elicitor systems (Dixon et al. 1981; Kombrink & Hahlbrock 1986). Genes encoding PAL were rapidly activated in

yeast-treated cassava suspension cells within 10-30 minutes and such early transcription has been reported in other plant cells (Hedrick et al. 1988). Furthermore, the rate of gene expression can be detected within minutes (Dixon et al. 1983). For instance, induction of transcription of genes for defence-related enzymes in bean suspension cells treated with fungal cell wall preparations occurs for PAL at 10 min and for chalcone synthase (CHS) as early as 5 min after elicitation (Hedrick et al. 1988). Differences in transcription rates among sets of elicitor-induced genes have been interpreted as evidence for more than one signalling pathway for their activation, or as responses of genes to different elicitor-active molecules (Somssich et al. 1989). Furthermore, the rapid early increase between 10-30 min in the transcripts of PAL and chalcone synthase (CHS) in nuclear transcript run-on analysis in yeast elicitor-treated alfalfa cells was interpreted as an activation of one or a sub-set of the genes. Other members of the gene families may be activated later with different kinetics, these included cinnamic acid 4-hydroxylase (C4H), chalcone isomerase (CHI), isoflavone reductase (IFR) and caffeic acid 3-*O*-methyltransferase (COMT) (Ni et al. 1996).

PAL transcription in cassava cells was maximal after 9 h of elicitation. In *Daucus carota* suspension cells elicited with proteins from *Pythium aphanidermatum* a similar maximum time point for PAL transcription was observed, however, unlike cassava cells, cell death also occurred (Koch et al. 1998). No PAL expression was detected in cassava leaves. This was probably due to only a minority of cells in the leaves expressing PAL, which as a result remained below detection levels in the total leaf RNA. Certainly, experiments with the cassava PAL2 promoter driving the reporter gene GUS in transgenic cassava showed only localised expression in the xylem parenchyma and meristematic cells in leaves (H Li and JR Beeching,

unpublished results). On the other hand, undifferentiated cassava suspension cells probably express PAL uniformly.

Pretreatment of parsley suspension cells with SA potentiated the elicitation of accumulation of phenylpropanoid products (Thulke & Conrath 1998). Furthermore, the important role of defence response potentiation was supported by experiments with cucumber seedlings with SA and other analogues which enhanced the activity of both chitinase and peroxidase in the systemic resistant tissue upon *Colletotrichum lagenarium* infection (Siegrist et al. 1994). A conditioning event occurred in cassava cells pre-treated for 24 h with salicylic acid; PAL was induced by a low concentration (5 μg/ml) of yeast elicitor, which would not otherwise have induced significant amounts of PAL mRNA. Similarly, PAL genes displayed SA-dependent potentiation of low-dose elicitation in parsley cells (Thulke & Conrath 1998). SA plays a key role in systemic acquired resistance (SAR), in which prior exposure to a pathogen enables the plant to mount a more effective defensive response upon subsequent exposure to the same or other pathogens (Hunt et al. 1996).

In cassava roots after post-harvest physiological deterioration (PPD) subsequent microbiological deterioration occurred (Beeching et al. 1998). UV-light fluorescence at the part adjacent to the wound site and the fungal-infected area, suggested accumulation of coumarins. A phenolic compound which was not scopoletin, but which ran at the same Rf as this coumarin, has been implicated as responsible for antimicrobial activity in cassava roots under PPD during the deterioration response (Taniguchi & Data 1984). Phenolic compounds, including scopoletin, scopolin, catechin, and esculetin, accumulated in wounded cassava tissues and deteriorating roots (Beeching et al. 1999; Tanaka et al. 1983b). Furthermore, in fresh cassava roots and leaves and under mealybug attack some

flavonoids (e.g kaempferol-3-*O*-rutinoside and rutin) were present and also increased in amount (Calatayud et al. 1994; Prawat et al. 1995). While terpenoid compounds were detected in cassava cell suspensions, some of which increased under elicitation, it did not prove possible to identify them. Twenty-two diterpenic stress metabolites, most of which were novel, were isolated and identified from cassava root tissues damaged by cutting or by fungal infection. The metabolites were classified into four families, *ent*-beyerane (10 components), *ent*-pimarane (9 components), *ent*-atisane (2-components) and *ent*-kaurane (1-component) (Sakai & Nakagawa 1988). Diterpenes have been reported as antimicrobial compounds in other members of the *Euphorbiaceae* family. For instance, casbene synthase catalyzes the cyclization of geranylgeranyl diphosphate to casbene, a diterpene phytoalexin with antibacterial and antifungal activity that is produced by seedlings of castor bean (*Ricinus communis* L.) in response to attack by *Rhizopus stolonifer* (Lee & West 1981).

Correlation between resistance to pathogen attack and increase of phenylpropanoid metabolism following infection or elicitor challenge has been found in many plants (Corchete et al. 1993; Butland et al. 1998). However, like cassava suspension cells that showed an induction of PAL and no phytoalexin production, it is a phenomenon that had been observed in other plants (R.A. Dixon, personal communication). In contrast to cassava, alfalfa suspension cells challenged with the same yeast elicitor preparation induced isoflavonoid phytoalexin production (medicarpin) (Dixon et al. 1995). In other plants yeast elicitor induced the production of isoflavonoids and alkaloids some of which were antimicrobial (Barz & Mackenbrock 1994; Borejsza-Wysocki et al. 1999; Schumacher et al. 1987). Cassava as a plant does not produce alkaloids and the flavonol rutin, though present,

did not show antimicrobial activity **(Figure 5.35)**. However, kaempherol-3-*O*-rutinoside was not tested, which has been reported to shown antimicrobial and antioxidant activity (El-Gammal & Mansour 1986; Haraguchi et al. 1997; Yamasaki et al. 1997).

Phenolic compounds were identified in cassava cells and leaves, including scopoletin, scopolin, kaempferol-3-*O*-rutinoside, and rutin. Some of these compounds had been previously reported to increase during wounding, PPD or other with defence responses of cassava (Calatayud et al. 1994; Tanaka et al. 1983a; Wheatley & Schwabe 1985). Although, none of these phenolic compounds gave a clear increase after elicitation it may be significant that scopoletin was reported to increase in cassava roots after wounding and was localized surrounding the vascular tissue (Tanaka et al. 1983a). Furthermore, scopoletin has been implicated as a phytoalexin in another member of *Euphorbiaceae* family, (*Hevea brasiliensis*) infected with *Microcyclus ulei* (García D et al. 1995). Recently, scopoletin has also been labelled as a phytoanticipin, in that it may be a defence compound mobilized from preformed precursors (Gutiérrez-Mellado et al. 1996).

Results from this chapter provide another example of the broad specificity of yeast elicitor, which in other plant suspension cultures also induces phenylpropanoid metabolism (Barz & Mackenbrock 1994; Kervinen et al. 1998; Wojtaszek et al. 1997). While secondary metabolites were detected in cell cultures and leaves, they were not abundant in the extracellular medium. In several reports the majority of the compounds identified from cells were also found in considerable amount in the extracellular medium (Sharan et al. 1998; Wink 1994). For instance, in tobacco suspension cells treated with methyl jasmonate up to 90% scopoletin accumulated extracellularly, but this effect was not observed in cells elicited with *Fusarium solani*

or in control (un-elicited) cells (Sharan et al. 1998). While the methods used here were capable of detecting a range of secondary metabolites in cassava cell suspensions and leaves and of identifying some of these, they were essentially qualitative rather than quantitative. This was largely due to the high variability obtained by HPLC quantification. Therefore, it was often difficult to determine with confidence whether there were differences in abundance of secondary metabolites between elicited and control cells, and changes over the time course of elicitation.

In general, germination and germ-tube elongation in *T. harzianum* were more sensitive to the four phenolic compounds tested than either *F. oxysporum* or *F. solani*. This may reflect that *T. harzianum* is a saprotroph, while the other two fungi are cassava-adapted pathogens. The different sensitivities of *T. harzianum* and *F. avenae* on the HPTLC bioassays revealed different inhibition zones; this underline the desirability of employing several organisms to show the potential range of antimicrobial compounds in a plant. Work on the fungitoxic effect of scopoletin, confirms this compound as a potential antimicrobial compound (Ahl Goy et al. 1993; Breton et al. 1997; García D et al. 1995; Giesemann et al. 1986; Valle et al. 1997). However, the efficacy of any antimicrobial agent depends upon it being at an appropriate concentration *in vivo*. A general rule was stated that any compound that not present any appreciable activity (e.g. 50 % inhibition of fungal-germ tubes) at more than $10^{-4}$ M is unlikely to have a key role in resistance (Mansfield 2000). In deteriorating cassava roots scopoletin was detected between 130 – 2,500 ppm (H Buschmann & J R Beeching, unpublished), and the highest concentration found here was 1,000 ppm. The average reduction of germination of *F. oxysporum* or *F. solani* observed here was less than 10 %, so it is possible that higher concentrations, which are nearer the maximum observed *in vivo*, are necessary to detect a significant effect

on pathogen germination. Of the phenolic compounds tested, ferulic acid proved to be the most effective as an inhibitor of germination and germ tube elongation, and this effect was shown not to be due to acidic pH. Increase in scopoletin up to 35 nmol/g FW and scopolin to 4 nmol/g FW in the hybrid *Nicotiana glutinosa* x *Nicotiana debneyi* leaves has been associated with resistance against *Cercospora nicotianae*, *Phytophthora parasitica* var. *nicotianae* (Goy et al. 1993). Neither in cells nor in leaves that amount of scopoletin was reached after elicitation, which ranges were around 0.036-0.048 nmol/g FW in control cells and 0.004-0.068 nmol/g FW in elicited cells. Leaves contained 0.253-2.77 nmol/g FW in control leaves and 1.15-4.21 nmol/g FW in elicited leaves. By contrast scopolin in leaves after 96 h reached between 4.032 and 6.003 nmol/g FW **(Tables 5.1, 5.2)**. By contrast to cassava, rubber tree accumulated up to 2 mM of scopoletin and this concentration was able to strongly inhibit germ tube elongation and conidial germination of their leaf pathogens including *C. gloeosporioides* and *Corynespora cassiicola* (García D et al. 1995). Additionally, 4nmol/ g FW of scopolin had been presented as sufficient to inhibit germ tube of three different fungi in *Nicotiana glutinosa* x *Nicotiana debneyi* (Goy et al. 1993). None of the four phenolic compounds tested inhibited mycelia growth of the three fungi despite their effect on germination and germ tube elongation. This may be due to the differential sensitivity of the fungi to the phenolics at different stages of their life cycles. However, there are also differences between the experimental methods that may affect the accessibility of the phenolics to the fungi. In the disc assay the fungi are grown on solid media, and there may be chemical interactions between the phenolics and the components of the agar media that limit their diffusion through the media and their accessibility to the fungi. Certainly, results from such experiments should be interpreted with caution (Cole 1994).

Peroxidases and tyrosinases can oxidise phenols to more reactive quinones, which have been shown to be significantly more fungitoxic that the phenols from which they are derived (Oku et al. 1975; Le Tourneau et al. 1957). Also, scopoletin degradation may produce new products, which possess higher toxicity thus enhancing its potential role in defence (Breton et al. 1997). Scopoletin and esculetin were shown to be readily oxidised by peroxidase and esculin by tyrosinase. Peroxidase requires $H_2O_2$ as an oxygen donor, which was shown to be produced in elicited cassava cells in Chapter 4. The results, presented here, showed that in the presence of a high concentration of $H_2O_2$, esculetin completely inhibited germination of *T. harzianum*, thereby considerably enhancing the toxicity of this compound. However, the oxidation of scopoletin did not increase its toxicity towards the three fungi tested.

The phenolic compounds tested and their oxidation products showed limited toxicity towards the three fungi tested. However, it is important to remember that *in vivo* secondary metabolites do not act in isolation, rather it is their cumulative and synergistic effects with each other and with other cellular constituents, such as enzymes and ROS, that may provide the total and effective defensive arsenal against most of the micro-organisms that a plant encounters. This provides what has been described as a defensive "soup" or more elegantly as a multi-component defence mechanism (Cole 1994). This was confirmed here, in that cell extracts of cassava cells showed significant antifungal activity compared to the pure phenolic compounds. However, TLC separation of these extracts revealed bands, which did not correspond to any of the reference compounds that were also antifungal. Whether these bands were individual compounds and what their identities were was not determined.

# CHAPTER 6

# GENERAL DISCUSSION AND FUTURE WORK

The research described in this thesis is the first in depth study of key strategies adopted by cassava suspension cells in response to microbial elicitation. Initially, a cassava suspension cell system was established and optimised. A considerable effect of auxin concentration was observed on the capacity of cassava suspension cells to trigger defence responses. At high auxin concentration in the growth medium an oxidative burst occurred but PAL activity was not induced. Investigations with tobacco cells revealed evidence that an oxidative burst is not necessary or sufficient for the activation of phenylpropanoid metabolism (Dorey et al. 1999). This auxin effect has been observed in other plants (Dixon & Fuller 1976). The system was optimised at 2 mg/l of 2,4-D in the growth medium in which an oxidative burst and PAL expression was observed. All subsequent experiments were carried out in the mid point of the exponential phase of cell growth as this point was shown to be critical in allowing optimal defence-responses in cultured plant cells (Eilert 1987). These observations stress the need for caution in designing and interpreting studies *in vitro* on host-pathogen interactions

The study has focussed on the modulation of reactive oxygen species and the activation of phenylpropanoid metabolism. Early defence responses such as alkalinisation of the medium pH and an early and transient production of hydrogen peroxide occurred following elicitation. Such responses do not require gene transcription and thus represent the earliest possible response of the plants against pathogens (Godiard et al. 1994). Yeast elicitor was chosen on the basis of the ability to induce maximum early responses (alkalinisation of the medium and oxidative burst). A broad specificity to glucan cell wall elicitors such as with the glucan from *Colletotrichum lindemuthianum*, which also triggered early responses in cassava was observed. In many fungi including *C. lindemuthianum* the principal component of the cell-wall is β-1,3-glucan.

Disappointingly it did not prove possible to clone a xanthine oxidase from cassava. A possibility to clone XO may be envisaged now with the cloning of aldehyde oxidase, another plant MoCo enzyme with a considerable part of its amino acid sequence highly similar to XO (Ori et al. 1997; Sarhan et al. 1982). However, the degree of intraspecific similarities between regions of these two genes would first have to be shown to be higher than the interspecific similarities of XO genes.

Increases in peroxidase levels in response to elicitation at the transcriptional and translational level were described in this research in both suspension cells and *in planta*. It is suggested here that possible roles of these peroxidases were strengthening of the plant cell-wall (e.g. *via* HRGP cross-linking) and oxidation of phenolic compounds to render them more toxic to the pathogens. The oxidised form of scopoletin was more toxic towards the phytopathogenic fungus *Fusarium solani*. A peroxidase isoform pI 3.6 was

present in cells, spent medium and leaves and showed high activity towards scopoletin. This is though to be the first report of a potential scopoletin-specific peroxidase isoform in cassava.

Since a cassava transformation system has been established in this laboratory, future studies on the roles of individual peroxidase isoforms could be carried out, using transgenic lines under or over expressing particular peroxidase isoforms, with a view towards generating lines with enhanced defence responses. Since the majority of the peroxidase isoforms detected were anionic, whose role in lignification has been pointed out in other plants (Dean et al. 1994) it is proposed that enhanced lignification may be a major component of the cassava defence response. Therefore, quantification of lignin would be of interest in order to elucidate further protection events after elicitation.

Steady state levels of catalase transcript in cells were down-regulated in response to yeast elicitor. It is proposed here that such down-regulation may play an important role in defence by facilitating hydrogen peroxide accumulation. Such hydrogen peroxide production could play several roles in plant defence. For example, as a signal triggering changes in gene expression at the level of the cell-wall strengthening and possibly as toxic molecules contributing both to host cell death and direct killing of pathogens (Peng & Kuc 1992; Mehdy 1994). Therefore, it would be interesting to test the toxicity of hydrogen peroxide at levels comparable to that found in suspension cells during the oxidative burst after yeast elicitation (10.5 μM) in *in vitro* bioassays with fungal and bacterial pathogens.

Although activation of phenylpropanoid metabolism was apparent, based on rapid and high induction of PAL, the major phenolics were preformed and had low antimicrobial activity. This might suggest alternative defence strategies or possibly a rapid turnover of these compounds. At 50 μg/ml of yeast elicitor a maximum expression of PAL gene(s) was observed 9 h after elicitation, with a subsequent decline of the expression at higher concentrations of yeast, suggesting the existence of a receptor in the cassava plasma membrane and subsequent receptor-saturation. The rapid expression of key enzymes in the phenylpropanoid pathway after elicitation or stress was interpreted as an activation of one or a sub-set of the involved genes (Ni et al. 1996). Other members of the gene families may be activated later with different kinetics. In addition, expression of PAL was shown to be potentiated by salicylic acid pre-treatment. Salicylic acid is widely proposed to function as a signal molecule which may directly induce SA-responsive genes and may act in concert with elicitor-inducible intracellular signalling components to potentiate activation of defence-related genes (Thulke & Conrath 1998). An extension of this work in cassava would be to include other defence-related genes, and salicylic acid analogues (e.g. 4-Chloro-SA, 3,5-dichloro-SA) to study further defence signal transduction in cassava.

It has been stated that any compound which lacks appreciable activity (e.g. 50 % inhibition of fungal germ-tubes) at more than $10^{-4}$ M is unlikely to play a major role in resistance (Mansfield 2000). However, in *in vitro* bioassays it is not possible to determine the actual concentration of the compounds taken up by the pathogen (Hammerschmidt 1999). However, none of these compounds was accumulated to $10^{-4}$ M *in planta* over 96 h after elicitation. In addition none

of the above mentioned compounds inhibited mycelial growth towards cassava phytopathogenic fungi.

Future studies should centre on the possible involvement *in planta* of these potential antimicrobial systems described above in response to manipulable fungal and bacterial diseases of cassava.

# APPENDIX 1

Analysis of variance (ANOVA) tables. See Chapter 3. Effects of different concentrations of 2,4-D on grown (fresh weight and dry weight) of cassava callus per petiole.

**FRESH WEIGHT**

| Source | DF | Sum of squares | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 0.067830 | 0.011305 | 12.63093 | 0.0000 |
| Effect error | 168 | 0.150364 | 0.000895 | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 1 | 25 | 0.094544 | 0.044776 |
| 1.5 | 25 | 0.073240 | 0.022477 |
| 2.0 | 25 | 0.039572 | 0.030363 |
| 2.5 | 25 | 0.053204 | 0.023134 |
| 3.0 | 25 | 0.051792 | 0.025342 |
| 3.5 | 25 | 0.047464 | 0.038002 |
| 4.0 | 25 | 0.032292 | 0.014548 |

**DRY WEIGHT**

| Source | DF | Sum of squares | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 0.000334 | 0.000056 | 13.51762 | 0.0000 |
| Effect error | 168 | 0.000693 | 0.000004 | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 1 | 25 | 0.005198 | 0.002815 |
| 1.5 | 25 | 0.005732 | 0.001650 |
| 2.0 | 25 | 0.002868 | 0.002090 |
| 2.5 | 25 | 0.002784 | 0.001699 |
| 3.0 | 25 | 0.01424 | 0.001090 |
| 3.5 | 25 | 0.003036 | 0.002555 |
| 4.0 | 25 | 0.003800 | 0.001802 |

**DUNCAN'S TEST**

**FRESH WEIGHT**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Level | 0.0945440 | 0.0732400 | 0.0395720 | 0.0532040 | 0.0517920 | 0.0474640 | 0.0322920 |
| 1 | | 0.011824 | 0.00101 | 0.000049 | 0.00030 | 0.00069 | 0.00154 |
| 1.5 | 0.011824 | | 0.002684 | 0.017902 | 0.59619 | 0.036779 | 0.00188 |
| 2.0 | 0.00101 | 0.002684 | | 0.752702 | 0.535247 | 0.351007 | 0.389613 |
| 2.5 | 0.000049 | 0.017902 | 0.752702 | | 0.867487 | 0.949879 | 0.335628 |
| 3.0 | 0.00030 | 0.59619 | 0.535247 | 0.867487 | | 0.609021 | 0.263612 |
| 3.5 | 0.00069 | 0.036779 | 0.351007 | 0.949879 | 0.609021 | | 0.314247 |
| 4.0 | 0.00154 | 0.000188 | 0.389613 | 0.335628 | 0.263612 | 0.314247 | |

**DRY WEIGHT**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Level | 0.0051984 | 0.0057320 | 0.0028680 | 0.0027840 | 0.0014240 | 0.0030360 | 0.0038000 |
| 1 | | 0.352864 | 0.00881 | 0.001064 | 0.000101 | 0.001005 | 0.014909 |
| 1.5 | 0.352864 | | 0.00090 | 0.000119 | 0.000154 | 0.00068 | 0.04448 |
| 2.0 | 0.00881 | 0.00090 | | 0.883731 | 0.62974 | 0.769904 | 0.416280 |
| 2.5 | 0.001064 | 0.00119 | 0.883731 | | 0.17895 | 0.989867 | 0.639592 |
| 3.0 | 0.000101 | 0.00154 | 0.62974 | 0.17895 | | 0.075377 | 0.001404 |
| 3.5 | 0.001005 | 0.00068 | 0.769904 | 0.989867 | 0.075377 | | 0.183447 |
| 4.0 | 0.014909 | 0.004448 | 0.416280 | 0.639592 | 0.001404 | 0.183447 | |

# APPENDIX 2

Clustal W multiple alignment of xanthine oxidase genes from *Bos Taurus* (B-tau), *Homo sapiens* (H-sap), *Gallus gallus* (G-gal), *Drosophila melanogaster* (D-mel) and *Aspergillus nidulans* (A-nid) showing the position of the PCR primers used in Chapter 4.

```
CLUSTAL W(1.60) multiple sequence alignment

                                                                        60
B-tau           ------------------------------------------------------------
H-sap           ------------------------------------------------------------
G-gal           ------------------------------------------------------------
D-mel           ------------------------------------------------------------
A-nid           ATGGCTCCCGGTGTCTTATTACAACCGTCGCAATCGGAGCTTGAGGCAGCTTCCCCGCCG

                                                                       120
B-tau           ------------------------------ATGACAGCAGATGAATTGGTT--TTCTTT
H-sap           ------------------------------ATGACAGCAGACAAATTGGTT--TTCTTT
G-gal           ------------------ATGGCTCCACCTGAGACAGGGGATGAGCTTGTT--TTCTTT
D-mel           ----------------------------ATGTCTAACTCGGTTTTGGTG--TTTTTC
A-nid           AAAGCTGCGGCCTCGTTATTGCAACTCACTGAAGAATGGGACGATACCATTCGCTTCTAT

                                                                       180
B-tau           GTGAATGGCAAAAAGGTGGTGGAGAAAAATGCAGATCCAGAAACAACCCTTTTGGCCTAC
H-sap           GTGAATGGCAGAAAGGTGGTGGAGAAAAATGCAGATCCAGAGACAACCCTTTTGGCCTAC
G-gal           GTGAATGGTAAAAAGGTGGTGGAAAAAGATGTGGACCCAGAAACAACTCTGCTGACCTAT
D-mel           GTGAATGGAAAGAAGGTGACCGAAGTGTCGCCTGATCCGGAGTGCACGCTCCTCACATTC
A-nid           CTTAATGGCACCAAGGTTATCTTGGACTCTGTTGACCCCGAAATCACATTGCTGGAATAC
                -----130f------>

                                                                       240
B-tau           CTGAGAAGAAAGTTGGGGCTGAGAGGAACCAAGCTGGGCTGTGGAGAAGGGGGCTGTGGG
H-sap           CTGAGAAGAAAGTTGGGGCTGAGTGGAACCAAGCTCGGCTGTGGAGAGGGGGGCTGCGGG
G-gal           CTACGAAGAAAACTGGGCCTATGTGGAACCAAACTGGGCTGTGGGGAAGGTGGATGTGGT
D-mel           CTGCGCGAAAAGCTGCGGCTGTGCGGAACGAAGTTGGGATGTGCGGAAGGCGGATGCGGC
A-nid           CTGAGGGGTA---TTGGCTTGACAGGGACAAAACTAGGGTGTGCAGAAGGTGGTTGTGGT

                                                                       300
B-tau           GCTTGTACAGTGATGCTTTCCAAGTATGATCGTCTCCAAGACAAGATCATCCACTTTTCT
H-sap           GCTTGCACAGTGATGCTCTCCAAGTATGATCGTCTGCAGAACAAGATCGTCCACTTTTCT
G-gal           GCTTGTACTGTTATGATATCCAAGTATGATCCCTTCCAGAAGAAAATCCTCCACCATACT
D-mel           GCCTGCACCGTGATGGTGTCCCGCCTAGACCGTCGGGCCAACAAGATACGTCACCTGGCG
A-nid           GCTTGCACGGTTGTCGTTTCGCAAATCAATCCGACTACCAAGAAGCTTTACCATGCCTCC

                                                                       360
B-tau           GCCAATGCCTGCCTGGCTCCCATCTGCACCTTGCACCACGTGGCTGTGACGACTGTGGAA
H-sap           GCCAATGCCTGCCTGGCCCCCATCTGCTCCTTGCACCATGTTGCAGTGACAACTGTGGAA
G-gal           GCCAATGCTTGCCTCTTCCCTATCTGTGCTCTGCATCATGTAGCTGTAACTACTGTCGAA
D-mel           GTCAACGCCTGCCTGACGCCAGTGTGCTCGATGCACGGATGTGCGGTGACCACTGTGGAG
A-nid           ATCAACGCCTGCATTGCGCCGCTGGTTGCTGTCGATGGGAAACACGTTATTACGGTGGAG

                                                                       420
B-tau           GGAATTGGAAGCACCAAGACGAGGCTGCATCCTGTGCAGGAGAGAATCGCCAAAAGTCAC
H-sap           GGAATAGGAAGCACCAAGACGAGGCTGCATCCTGTGCAGGAGAGAATTGCCAAAAGCCAC
G-gal           GGCATAGGAAACACAAAGTCCAGGTTGCACCCAGCCCAGGAGAGAATAGCAAAAAGTCAT
D-mel           GGTATAGGGAGCACCAAAACGCGTCTGCATCCGGTGCAGGAGCGACTGCCCAAGGCGCAC
A-nid           GGCATTGGGAACGTTAAGA---ATCCCCATGCGATTCAGCAACGGCTAGCAATCGGAAAC
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                                                                                480
B-tau           GGCTCTCAGTGTGGGTTCTGCACCCCCGGCATCGTCATGAGCATGTACACACTGCTTCGG
H-sap           GGCTCCCAGTGCGGGTTCTGCACCCCTGGCATCGTCATGAGTATGTACACACTGCTCCGG
G-gal           GGGTCCCAATGTGGCTTTTGCACTCCGGGCATTGTTATGTCCATGTACACATTGCTTCGA
D-mel           GGATCCCAGTGCGGCTTCTGCACGCCGGGCATTGTGATGTCCATGTACGCACTTCTGCGG
A-nid           GGAAGCCAGTGCGGTTTCTGTACACCGGGAATTGTGATGAGTCTCTATGCGCTCTTACGA
                                    ----------------------459f----->

                                                                                540
B-tau           AATCAGCCCGAGCCCACTGTTGAAGAAATCGAGGATGCTTTCCAAGGAAACTTATGCCGC
H-sap           AATCAGCCCGAGCCCACCATGGAGGAGATTGAGAATGCCTTCCAAGGAAATCTGTGCCGC
G-gal           AACAAACCCAAACCCAAAATGGAGGACATAGAAGACGCTTTCCAAGGGAACTTGTGTCGG
D-mel           AACGCGGAGCAACCCTCTATGCGAGACTTGGAGGTGGCATTCCAAGGTAACCTGTGCCGC
A-nid           AATGACCCTAAGCCCTCTGAGCATGCGGTGGAAGAAGCTTTTGACGGAAACCTTTGTCGC

                                                                                600
B-tau           TGTACAGGCTACAGACCCATCCTCCAGGGCTTCCGGACCTTTGC----------------
H-sap           TGCACAGGCTACAGACCCATCCTCCAGGGCTTCCGGACCTTTGC----------------
G-gal           TGTACAGGATACAGACCCATTCTGGAGGGATACAGGACTTTCGCTGTGGACTCAAACTGC
D-mel           TGCACCGGCTATCGACCCATTCTCGAGGGCTACAAGACGTTCAC----------------
A-nid           TGTACTGGATATCGACCGATTTTGGACGCTGCCCAAAGCTTTAC----------A--AGC
                --------557f----->

                                                                                660
B-tau           ------------------------------------------------------CAAGAAT
H-sap           ------------------------------------------------------CAGGGAT
G-gal           TGTGGCAAAGCAGCCAATGGCACTGGATGCTGTCATAGTAAGGGAGAAAACAGCATGAAT
D-mel           ------------------------------------------------------CAAGGAG
A-nid           C----------C--------------AATCGGCTGTGGCAAAGCTCGAGCAAACGGTGGC

                                                                                720
B-tau           GGTGGATGCTGTGGAGGAAATGGGAACAACCCAAACTGCTGCATGAACCAGAAGAAAGAC
H-sap           GGTGGATGCTGTGGAGGAGATGGGAATAATCCAAATTGCTGCATGAACCAGAAGAAAGAC
G-gal           GGGGGTTGCTGTGGAGGAAAAGCCAATGGCCCAGGCTGCTGCATGAATGAAAAAGAGAAC
D-mel           TTTGC---CTGCGGAATGGGCGAGAAGTGCTGCAAAGTTAGTGGGAAAGGATGTGGAACC
A-nid           TCTGGATGCTGTATGGAAGAACAGAAAGGCACAAATGGATGTTGCAAAGGGTCTTCCGAG

                                                                                780
B-tau           CACACCCAGGTTACT--CTCTCACCATCTTTATTCAACCCAAAGGAGTTCATGCCCCTGG
H-sap           CACTC---AGTCAGC--CACTCGCCATCTTTATTCAAACCAGAGGAGTTCACGCCCCTGG
G-gal           GTGACA-ATGATGTC--CTCCAGCCTGTTTGATTCTTCC-----GAGTTCCAGCCATTGG
D-mel           GATGC----GGAGAC--CGATGACAAGCTC--TTCGAGCGCAGCGAATTCCAGCCCTTGG
A-nid           GAGACTACCGAAGACGTTAAGCACAAGTTTGCGTCTCCC-----GACTTTATTGAGTACA

                                                                                840
B-tau           ATCCTACCCAGGAACCCATCTTCCCTCCAGAGTTGCTGAGGCTGAAAGACGTTCCACCGA
H-sap           ATCCAACCCAGGAGCCCATTTTTCCCCCAGAGTTGCTGAGGCTGAAAGACACTCCTCGGA
G-gal           ACCCCACACAGGAACCAATCTTCCCACCAGAGTTAATGACTCAGAGAAACAAGGAGCAGA
D-mel           ATCCCAGCCAGGAACCCATCTTCCCACCGGAACTTCAGCTGAGTGACGCCTTCGATTCGC
A-nid           AACCAGACACGGAACTAATATTTCCTCCGTCGCTCTGGAAACA---------CGAGTTGC

                                                                                900
B-tau           AGCAGCTGCGTTTTGAAGGGGAGCGTGTGACCTGGATCCAAGCCTCCACCTTGAAGGAGT
H-sap           AGCAGCTGCGATTTGAAAGGGAGCGTGTGACGTGGATACAGGCCTCAACCCTCAAGGAGC
G-gal           AGCAGGTGTGTTTCAAAGGTGAACGCGTGATGTGGATCCAGCCTACGACTCTCCAGGAGC
D-mel           AGAGTTTGATCTTTAGTTCGGATAGGGTGACCTGGTATCGTCCCACCAATCTGGAGGAGC
A-nid           GCCCTCTCGCTTTTGGTAACAAGAGAAAGAAGTGGTATCGGCCGGTCACCGTACAGCAGC

                                                                                960
B-tau           TGCTGGACCTCAAAGCTCAGCATCCCGAGGCCAAGCTGGTGGTGGGGAACACAGAGATCG
H-sap           TGCTGGACCTCAAGGCTCAGCACCCTGACGCCAAGCTGGTCGTGGGGAACACGGAGATTG
G-gal           TGGTGGCACTCAAATCCCAATATCCCAATGCCAAGCTTGTTGTGGGGAACACAGAAGTGG
D-mel           TGCTTCAGCTGAAGGCCAAACATCCGGCTGCCAAGCTGGTCGTGGGCAATACGGAAGTGG
A-nid           TCCTGGAGATCAAGAGTATTCATCCTGATGCAAAATTGATAGGTGGCAGCACCGAGACGC
```

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                                                                    1020
B-tau           GCATTGAGATGAAGTTTAAGAATCAGCTATTTCCTATGATCATCTGCCCAGCCTGGATCC
H-sap           GCATTGAGATGAAGTTCAAGAATATGCTGTTTCCTATGATTGTCTGCCCAGCCTGGATCC
G-gal           GTATTGAAATGAGGTTAAAGAACATGTTGTATCCAGTGATACTAGCACCAGCATGGATTC
D-mel           GCGTTGAGGTTAAGTTCAAGCACTTCCTCTACCCGCACCTCATCAATCCCACCCAGGTGA
A-nid           AGATCGAGATCAAATTCAAGCAGATGCGTTATGGAGCATCTGTCTACCTTGGAGACCTCG

                                                                    1080
B-tau           CTGAGCTGAATGCAGTGGAGCACGGACCTGAGGGGATCTCCTTTGGAGCTGCTTGTGCCC
H-sap           CTGAGCTGAATTCGGTAGAACATGGACCCGACGGTATCTCCTTTGGAGCTGCTTGCCCCC
G-gal           CTGAGATGAATGCTGTTCAACAGACTGAAACAGGGATCACCTTTGGCGCTGCCTGTACTT
D-mel           AGGAGCTGCTGGAGATCAAAGAGAACCAGGATGGCATTTACTTCGGTGCGGCTGTCAGTT
A-nid           CTGAACTCCGGCAATTTGCCTTTCATGACAACTACTTGGAAATTGGTGCCAACATTTCAT

                                                                    1140
B-tau           TGAGCTCTGTGGAAAAGACCCTGCTCGAGGCCGTTG--CCAAGCTTCCCACCCAGAAAAC
H-sap           TGAGCATTGTGGAAAAAACCCTGGTGGATGCTGTTG--CTAAGCTTCCTGCCCAAAAGAC
G-gal           TAAGCTCAGTAGAAGAGGTGCTGAGAAAAGCGGTGG--CAGAGCTTCCTTCTTACAAAAC
D-mel           TGATGGAGATCGATGCGCTTCTGCGGCAGAGAATCG--AGCTGCTGCCGGAATCGGAGAC
A-nid           TAACTGATCTTGAATCTGTCTGTGATCAGGCCATCGAGCGCTACGGCTCAGCTCGCGGCC

                                                                    1200
B-tau           GGAGGTGTTCAGAGGAGTCCTGGAGCAGCTGCGCTGGTTCGCTGGGAAGCAGGTCAAGTC
H-sap           AGAGGTGTTCAGAGGGGTCCTGGAGCAGCTGCGCTGGTTTGCTGGGAAGCAAGTCAAGTC
G-gal           TGAGATTTTCCAGGCTGCCCTGGAACAGCTGCGGTGGTTTGCAGGGCCACAGATCAGGAA
D-mel           CAGATTGTTCCAGTGCACCGTGGATATGCTTCACTACTTTGCCGGCAAGCAGATCCGCAA
A-nid           AGCCCTTTGCCG--CTATAAAGAAACAGCTTCGCTACTTTGCTGGGAGACAGATCAGAAA

                                                                    1260
B-tau           TGTGGCGTCCCTTGGAGGGAACATCATCACAGCCAGCCCCATCTCTGACCTCAACCCTGT
H-sap           TGTGGCGTCCGTTGGAGGGAACATCATCACTGCCAGCCCCATCTCCGACCTCAACCCCGT
G-gal           TGTTGCAGCTCTTGGTGGGAACATAATGACAGCAAGCCCAATCTCTGACTTAAACCCTGT
D-mel           CGTCGCCTGTTTGGGTGGAAACATCATGACCGGCAGTCCCATTTCCGATATGAATCCTGT
A-nid           TGTGGCTTCGCCAGCTGGGAACCTGGCCACTGCATCCCCGATATCCGATCTCAACCCAGT

                                                                    1320
B-tau           GTTCGTGGCCAGTGGGACCAAGCTGACCATCG-------TGTCCAGAGGCACC-----AG
H-sap           GTTCATGGCCAGTGGGGCCAAGCTGACACTAG-------TGTCCAGAGGCACC-----AG
G-gal           GTTAATGGCAAGTGGAAGCAAACTGACTCTGA-------TATCAATGGAGGGC-----AA
D-mel           GCTCTCGGCAGCAGGAGCTCAACTGGAGGTGGCCAGTTTTGTGGATGGAAAGCTCCAAAA
A-nid           TTTTGTCGCTACAAACACGACTCTTGTCGCCA-------GGTCGTTAGATAAG-----GA

                                                                    1380
B-tau           AAGAACAGTTCCAATGGACCACACCTTCTTCCCCAGCTACAGAAAGACCCTGCTGGGTCC
H-sap           GAGAACTGTCCAGATGGACCACACCTTCTTCCCTGGCTACAGAAAGACCCTGCTGAGCCC
G-gal           GAGGACTGTCATGATGGATGAGAAGTTTTTCACTGGATACAGAAAAACTATAGTTAAGCC
D-mel           GAGATCAGTTCACATGGGAACTGGGTTCTTCACTGGCTATCGCAGGAATGTTATCGAAGC
A-nid           AACCGAGATTCCAATGACACA---GTTCTTCAGAGGTTACCGATCTACGGCCCTTCCGCC

                                                                    1440
B-tau           AGAGGAGATACTGCT--CTCCATTGAGATCCCCTACAGC-AGGGAGGATGAGTTTTTCTC
H-sap           GGAGGAGATACTGCT--CTCCATAGAGATCCCCTACAGC-AGGGAGGGGGAGTATTTCTC
G-gal           TGAGGAAGTGCTGCT--CTCTGTTGAGATACCCTACAGC-AAGGAGGGTGAATACTTCTC
D-mel           CCACGAGGTGCTGCT--GGGCATCCACTTTCGGAAGACC-ACTCCGGACCAGTATATCGT
A-nid           AGACGCTATCATTTCAAGTCTACGTATACCTACCGCATCTGAGAAAGGCGAGTATTTGCG

                                        <-----1466r----------
                                          <----1469r--------
                                                                    1500
B-tau           AGCATTCAAGCAGGCAAGCCGGAGAGAAGATGACATAGCCAAGGTGACCTGCGGCATGAG
H-sap           AGCATTCAAGCAGGCCTCCCGGAGAGAAGATGACATTGCCAAGGTAACCAGTGGCATGAG
G-gal           AGCTTTCAAGCAGGCTTACCGTAGGGAGGATGACATTGCTATTGTGACCTGTGGAATGAG
D-mel           TGCTTTTAAGCAGGCCAGAAGAAGGGATGATGACATAGCCATCGTAAATGCCGCAATAAA
A-nid           GGCTTATAAACAATCTAAGAGGAAGGATGATGACATTGCAATTGTGAATGCCGCTTTGCG
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                                                                    1560
B-tau           AGTCCTGTTCCAGCCAGGAAGCATGCAGGTAAAGGAGCTAGCCCTCTGCTATGGCGGAAT
H-sap           AGTTTTATTCAAGCCAGGAACCACAGAGGTACAGGAGCTGGCCCTTTGCTATGGTGGAAT
G-gal           AGTCCTGTTCCAACATGGCACCAGCCGAGTGCAGGAGGTCAAACTAAGCTATGGCGGAAT
D-mel           CGTTCGCTTTGAGGAAAAATCCAACATTGTGGCGGAGATCTCGATGGCTTTTGGTGGAAT
A-nid           GGTTTCACTCTCGTC---ATCGAACGATGTCACTAGCGTGAGCCTAGTTTTTGGCGGTAT
                                                                  -----1563f-

                                                                    1620
B-tau           GGCGGACAGAACCATCTCAGCCCTCAAGACC-ACGCAGAAGCAGCTGTCAAAGTTCTGGA
H-sap           GGCCAACAGAACCATCTCAGCCCTCAAGACC-ACTCAGAGGCAGCTTTCCAAGCTCTGGA
G-gal           GGCTCCTACAACCATCCTGGCACTAAAAACTTGCCGAGAGCTCGCTGGCAGAGA-CTGGA
D-mel           GGCACCAACCACAGTGCTCGCTCCTCGAACTTCCCAACTGATGGTTGGGCAGGA-GTGGA
A-nid           GGCGCCTTTGACGGTATCTGCGCGGAACGCGGAGGCCTTCCTCACCGGGAAGAAGTTCAC
                -->

                                                                    1680
B-tau           ATGAGAAGCTGCTACAGGACGTGT-GTGCAGGCCTGGCGGA-AGAACTGTCCTTATCCCC
H-sap           AGGAGGAGCTGCTGCAGGACGTGT-GTGCAGGACTGGCAGA-GGAGCTGCATCTGCCTCC
G-gal           ATGAGAAGCTGCTGCAGGATGCCT-GCCGCTTGCTGGCAGG-TGAGATGGACCTGTCTCC
D-mel           GCCACCAGCTCGTGGAGCGCGTG--GCGGAGAGCTTGTGCACGGAGCTGCCTTTGGCTGC
A-nid           TGATCCGGCAACTCTAGAAGGTACTATGGGTGCTTTGGAGCAGGATTTCAACCTGAAGTT

                                                                    1740
B-tau           AGACGCCCCTGGAGGCATGATTGAATTCCGACGCACCCTCACCCTCAGCTTCTTCTTCAA
H-sap           CGATGCCCCTGGTGGCATGGTGGACTTCCGGTGCACCCTCACCCTCAGCTTCTTCTTCAA
G-gal           TTCTGCACCTGGTGGGATGGTGGAATTCCGACGCACGCTCACACTCAGCTTCTTCTTCAA
D-mel           CTCCGCTCCGGGTGGCATGATCGCCTATCGTCGAGCTCTGGTGGTGAGCCTGTTCTTCAA
A-nid           TGGTGTTCCAGGTGGTATGGCGACTTACCGAAAGTCACTTGCTCTCGGGTTCTTCTACCG

                                                                    1800
B-tau           GTTCTACCTGACAGTACTGAAGAAACTGGGC---AAGGACTCGAAAG------ATAAGTG
H-sap           GTTCTACCTGACAGTCCTTCAGAAGCTGGGCCAAGAGAACCTGGAAG------ACAAGTG
G-gal           ATTCTATCTGACCGTCCTTCAGAAGCTGAGC---AAGGACCAGAATGGCCCTAACAATCT
D-mel           GGCCTATCTGGCCATTTCCCTGAAGCTGAGC----AAGTCAGGGATC------ACATCTT
A-nid           ATTCTACCATGACGTATTATCACAGATCG------AGGCCAGGAGTA------GTGATCT

                                                                    1860
B-tau           TGGTAAACTGGACCCCAC---CTACACCAGCGCCACTTTACTCTTTCAGAAAGACCCTCC
H-sap           TGGTAAACTGGACCCCAC---TTTCGCCAGTGCAACTTTACTGTTTCAGAAAGACCCCCC
G-gal           GTGTGAGCCTGTCCCTCCAAACTACATCAGTGCTACGGAGTTGTTTCACAAAGATCCCAT
D-mel           CCGATGCTCTGCCACCGG--AGGAGCGAAGTGGTGCCGAGACATTCCACACACCAGTACT
A-nid           GGATAACAGTGTGGTCGC----T--GAAATTG--AGCGAGCAATTTCA---------ACT

                                                                    1920
B-tau           AGCCAACATCCAGCTCTTCCAAGAGGTGCCCAATGGTCAGTCCAAGGAGGACACAGTGGG
H-sap           AGCCGATGTCCAGCTCTTCCAAGAGGTGCCCAAGGGTCAGTCTGAGGAGGACATGGTGGG
G-gal           TGCAAGCACCCAGCTCTTCCAAGAAGTGCCCAGGGGGCAGTTGGTGGAAGATACAGTGGG
D-mel           CAAAAGTGCCCAGCTCTTCGAGCGCGTCTGCAGCGATCAACCCATCTGTGATCCCATTGG
A-nid           GGAGAGAA---AGAT-----AACGAGGCTTCAGCGGCTTACCAGCAGAGGGTTC--TAGG

                                                                    1980
B-tau           CCGGCCCCTGCCCCACCTGGCCGCGGCCATGCAAGCCTCTGGGGAGGCCGTATACTGTGA
H-sap           CCGGCCCCTGCCCCACCTGGCAGCGGACATGCAGGCCTCTGGTGAGGCCGTGTACTGTGA
G-gal           CCGGCCTTTGGTGCACCTTTCAGCAGCAAAACAAGCATGCGGGGAGGCCGTTTACTGTGA
D-mel           GAGACCAAAAGTTCATGCGGCTGCTTTGAAACAGGCCACTGGTGAAGCTATCTACACAGA
A-nid           CCGAGCCGGGCCGCACCTCTCGGCATTGAAACAGGCTACCGGAGAAGCTCAGTACACCGA

                                                                    2040
B-tau           CGACATCCCTCGCTACGAGAATGAGCTGTTCCTCCGGCTGGTCACCAGCACCCGGGCCCA
H-sap           CGACATTCCTCGCTACGAGAATGAGCTGTCTCTCCGGCTGGTCACCAGCACCCGGGCCCA
G-gal           TGACATCCCACACTATGAGAACGAGCTGTATCTCACACTGGTGACCAGCACCCAAGCCCA
D-mel           TGACATTCCCCGCATGGATGGTGAAGTTTATCTGGCCTTTGTCCTTAGTACCAAGCCACG
A-nid           CGACATTCCCGCGCAAAAGAACGAACTGTACGGATGTATGGTTTTGTCCACAAAGGCTCA
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                                                                    2100
B-tau           CGCCAAGATCAAGTCCATTGATGTTTCAGAAGCTCAGAAAGTGC-CAGGATTTGTTTGCT
H-sap           CGCCAAGATCAAGTCCATAGATACATCAGAAGCTAAGAAGGTTC-CAGGGTTTGTTTGTT
G-gal           TGCTAAAATCCTTTCTATAGATGCATCTGAAGCCCAGAGTGTCC-CTGGGTTTGTGTGTT
D-mel           TGCCAAGATCACCAAGCTGGATGCCAGTGAAGCTCTGGCCCTGGACGGAGTGCATCAGTT
A-nid           CGCCAAGCTGCTCAGTGTCAACACAGAAGCCGCTTTGGAAATTC-CTGGTGTCATCGATT

                                                                    2160
B-tau           TTCTCTCTGCTGATGATATTCCCGGGA--GTAATGAAACCGGA---CTCTTTAA----TG
H-sap           TCATTTCCGCTGATGATGTTCCTGGGA--GTAACATAACTGGA---ATTTGTAA----TG
G-gal           TTGTCTCTGCTAAGGATGTTCCTGGCA--GTAACATCACTGGC---ATCGCTAA----TG
D-mel           CTTTTGCTACAAGGACTTAACGGAGCACGAGAACGAAGTGGGACCCGTCTTTCA----TG
A-nid           ATGTAGATCATAAAGACCTTCCATCACCGAGAGCGAACTGGTGGGGTGCTCCAAATTGTG


                                                                    2220
B-tau           ATGAGACAGTCTTTGCGAAGGATACGGTGACTTGTGTTGGTCACATCATTGGTGCTGTGG
H-sap           ATGAGACAGTCTTTGCGAAGGATAAGGTTACTTGTGTTGGGCATATCATTGGTGCTGTGG
G-gal           ATGAGACTGTCTTTGCTGAGGATGTGGTCACCTGTGTGGGTCACATCATTGGTGCAGTCA
D-mel           ATGAGCACGTCTTTGCCGCTGGAGAAGTGCATTGCTATGGTCAGATAGTGGGCGCCATAG
A-nid           ACGAGGTCTTCTTTGCTGTCGATAAAGTCACAACTGCCGGACAGCCAATTGGTATGATTC
                -----2176f----→

                                                                    2280
B-tau           TCGCTGACACCCCAGAACATGCAGAGAGAGCTGCCCATGTAGTGAAAGTCACCTATGAGG
H-sap           TTGCTGACACCCCGGAACACACACAGAGAGCTGCCCAAGGGGTGAAAATCACCTATGAAG
G-gal           TTGCAGACACACAGGAACATTCCAGGAGAGCAGCTAAAGCTGTGAAGATTAAGTATGAGG
D-mel           CTGCCGATAATAAGGCGTTGGCCCAAAGAGCCGCTCGGCTAGTGAAAGTGGAGTACGAGG
A-nid           TTGCAAACACAGCCAAGGCTGCAGAAGAGGGTGCAAGAGCCGTCAAAGTTGAATATGAAG

                                                                    2340
B-tau           ATCTTCCTGCCATTATC---ACAATTGAGGATGCTATAAAAAATAATTCCT--TTTATGG
H-sap           AACTACCAGCCATTATC---ACAATTGAGGATGCTATAAAGAACAACTCCT--TTTATGG
G-gal           AGCTCAAACCGATTGTC---ACAATTCAGGAAGCTATTGAGCAACAATCCT--TCATTAA
D-mel           AGCTGAGCCCGGTTATCGTGACCATAGAGCAGGCCATCGAGCTCAAGTCCTATTTCCCGG
A-nid           AATTGCCAGTAATCCTC---AGCATCGAAGAGGCAATCGAAGCGCAATCCT--TCTTCGA

                                                                    2400
B-tau           ATCTGAGCTGAAGATTGAG-AAAGGAGACCTCAAGAAGGGGTTTTCAGAAGCAGATAATG
H-sap           ACCTGAGCTGAAGATCGAG-AAAGGGGACCTAAAGAAGGGGTTTTCCGAAGCAGATAATG
G-gal           ACCTATAAAGAGGATTAAA-AAGGGAGATGTGAATAAAGGATTTGAAGAATCTGATCACA
D-mel           ACTACCCCCGATTCGTGACCAAGGGCAATGTGGAGGAGGCTTTATCCCAGGCGGATCACA
A-nid           ACACTTCCGTTATATCAAG-AATGGAGACCCGGAAAGCGCCTTCAGAGACGCTGACCATG

                                                                    2460
B-tau           TGGTTTCAGGTGAGCTGTACATTGGTGGCCAAGACCACTTCTACCTGGAGACTCACTGCA
H-sap           TTGTGTCAGGGGAGATATACATCGGTGGCCAAGAGCACTTCTACCTGGAGACTCACTGCA
G-gal           TTTTGGAAGGAGAGATGCACATTGGTGGACAGGAGCATTTCTATCTGGAAACTCACTGTA
D-mel           CTTTCGAGGGCACCTGTCGAATGGGCGGACAGGAGCACTTCTATCTGGAGACCCATGCTG
A-nid           TCTTTGAGGGTGTATCTCGAATGGGGGGCCAGGAACATTTTTATTTAGAAACACAAGCTT

                                                                    2520
B-tau           CCATTGCTATCCCGAAAGGCGAGGAAGGGGAGATGGAGCTCTTTGTGTCCACACAGAATG
H-sap           CCATTGCTGTTCCAAAAGGCGAGGCAGGGGAGATGGAGCTCTTTGTGTCTACACAGAACA
G-gal           CTTTGGCTGTGCCCAAGGGGGAAGATGGTGAGATGGAACTCTTTGTGTCAACCCAAAACT
D-mel           CATTGGCCGTACCTC---GTGACAGCGATGAGCTGGAACTCTTTTGCTCCACGCAGCATC
A-nid           GTGTGGCTATCCCTAAAGCAGAAGACGGCGAAATGGAAATCTGGAGCAGTACCCAGAATC

                                                                    2580
B-tau           CCATGAAAACCCAGTCCTTTGTTGCAAAAATGTTGGGGGTTCCAGTAAACCGGATTTTGG
H-sap           CCATGAAGACCCAGAGCTTTGTTGCAAAAATGTTGGGGGTTCCAGCAAACCGGATTGTGG
G-gal           TAATGAAGACACAGGAGTTTACTGCTAGTGCACTGGGAGTCCCATCAAATCGCATCGTGG
D-mel           CCTCGGAGGTGCAGAAGCTAGTGGCCCATGTAACCGCACTTCCTGCCCACCGTGTCGTCT
A-nid           CGACGGAAACGCAATCATATGTAGCACAGGTTACTGGCGTGGCTGCCAACAAGATCGTGT
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                                                                    2640
B-tau           TCCGAGTGAAGAGAATGGGAGGAGGCTTTGGAGGGAAGGAGACCCGGAGCACCCTGGTGT
H-sap           TTCGAGTGAAGAGAATGGGAGGAGGCTTTGGAGGCAAGGAGACCCGGAGCACTGTGGTGT
G-gal           TTCGAGTGAAAAGAATGGGTGGAGGATTTGGAGGAAAAGAGACTAGAAATACTATTCTGA
D-mel           GTCGTGCCAAGCGTTTGGGAGGCGGTTTCGGCGGCAAGGAGTCCAGAGGCATCTCCGTGG
A-nid           CAAGGGTCAAGCGCCTTGGAGGAGGCTTTGGTGGTAAAGAGACGCGGTCAGTCCAGCTCG

                                                                    2700
B-tau           CTGTGGCTGTGGCCCTGGCTGCATACAAGACTGGACACCCGGTGCGCTGCATGCTGGATC
H-sap           CCACGGCAGTGGCCCTGGCTGCATATAAGACCGGCCGCCCTGTGCGATGCATGCTGGACC
G-gal           CCACTGTAGTGGCTGTGGCAGCCTTCAAAACTGGCCGACCCGTAAGGTGCATGTTGGATC
D-mel           CCCTACCCGTTGCCCTGGCCGCCTATCGAATGGGTCGTCCTGTGCGCTGTATGTTGGATC
A-nid           CAGGTATATGCGCCACAGCAGCCGCAAAAGTCAGGCGTCCAGTGCGGTGTATGCTCAATC

                                                                    2760
B-tau           GTAATGAGGATATGCTGATAACTGGCGGCAGACACCCCTTCTTGGCCAGATACAAGGTTG
H-sap           GTGATGAGGACATGCTGATAACTGGTGGCAGACATCCCTTCCTGGCCAGATACAAGGTTG
G-gal           GAGATGAGGACATGCTGATAAGTGGTGGGAGACATCCTTTCCTGGGGAGGTACAAGGTTG
D-mel           GCGATGAGGACATGCTTATCACCGGCACCAGGCATCCCTTCCTCTTCAAATACAAAGTGG
A-nid           GCGATGAAGACATTGCGACTTCTGGACAGCGTCACCCATTCTATTGTAAATGGAAAGTCG

                                                                    2820
B-tau           GCTTCATGAAGACAGGGACGATTGTGGCTCTAGAGGTGGATCACTACAGCAATGCCGGGA
H-sap           GCTTCATGAAGACTGGGACAGTTGTGGCTCTTGAGGTGGACCACTTCAGCAATGTGGGGA
G-gal           GTTTCATGAAGAATGGAAAAATCAAGAGTTTGGAAGTCTCATACTACAGCAATGGGGGCA
D-mel           GCTTCACCAAGGAGGGTCTGATCACTGCCTGCGACATTGAGTGCTACAACAATGCCGGTT
A-nid           GGGTGACAAGGGAGGGCAAGCTGCTTGCACTTGATGCGGACGTGTACGCAAATGGTGGAC

                                                                    2880
B-tau           ACAGCCGGGACCTCTCTCACAGTATAATGGAACGAGCTCTATTCCACATGGACAACTGCT
H-sap           ACACCCAGGATCTCTCTCAGAGTATTATGGAACGAGCTTTATTCCACATGGACAACTGCT
G-gal           ACTCTGCAGACCTCTCTCATGGTGTCATGGATAGAGCCCTGTTACATTTGGATAACTCCT
D-mel           GGTCCATGGATCTGTCATTTTCGGTTCTTGAGCGCGCCATGTTCCACTTTGAGAATTGCT
A-nid           ATACACAGGATCTTTCAGGTGCTGTTGTGGAGCGAAGTCTTTCACACATTGACAACGTAT

                                                                    2940
B-tau           ATAAAATCCCCAACATCCGGGGCACTGGGCGGCTGTGCAAGACCAACCTGTCCTCCAACA
H-sap           ATAAAATCCCCAACATCCGGGGCACTGGGCGGCTGTGCAAAACCAACCTTCCCTCCAACA
G-gal           ACAACATCCCCAATGTCAGCATCATGGGCTTTATATGCAAGACCAACTTGTCTTCCAACA
D-mel           ACAGGATTCCCAACGTTCGCGTGGGTGGATGGGTCTGCAAGACGAACCTGCCCTCGAATA
A-nid           ATCGATTCCCGAACATTTACGTCCGGGGCAGGATATGCAAGACGAACACCGTCTCAAATA

                ←----2943r-----------------
                                                                    3000
B-tau           CGGCCTTCCGGGGCTTTGGGGGGCCCCAGGCTCTGTTCATTGCCGAGAACTGGATGAGCG
H-sap           CGGCCTTCCGGGGCTTTGGGGGGCCCCAGGGGATGCTCATTGCCGAGTGCTGGATGAGTG
G-gal           CAGCCTTCCGTGGCTTTGGAGGTCCCCAAGGAATGATGATTGCTGAGTGCTGGATGAGTG
D-mel           CGGCCTTCCGTGGATTTGGAGGACCACAAGGCATGTACGCCGGTGAGCATATCATCCGGG
A-nid           CGGCATTCCGGGGCTTTGGTGGCCCTCAAGGTCTCTTTTTTGCCGAGTCAATCATCTCAG
                --------2959r----→
                                                                    3060
B-tau           AAGTTGCAGTGACCTGTGGGCTGCC-TGCAGAGGAAGTGCGGTGGAAGAACATGTACAAA
H-sap           AAGTTGCAGTGACCTGTGGGATGCC-TGCAGAGGAGGTGCGGAGAAAAAACCTGTACAAA
G-gal           ACCTTGCTCGGAAGTGTGGCCTACC-ACCTGAGGAGGTACGGAAGATCAACCTGTATCAT
D-mel           ATGTGGCCCGGATA-GTGGGTCGCGATGTGGTGGATGTGATGCGGCTGAACTTCTACAAG
A-nid           AAGTCGCA-GATCATCTAGACCTTCAGGTGGAACAGCTCCGGATACTCAACATGTACGAA

                                                                    3120
B-tau           GAAGGGGACCTGACCCACTTCAACCAGAAGCTTGAGGGGTTCAGCGTGCCCAGGTGCTGG
H-sap           GAAGGGGACCTGACACACTTCAACCAGAAGCTTGAGGGTTTCACCTTGCCCAGATGCTGG
G-gal           GAAGGAGACCTGACTCATTTTAACCAAAAACTGGAGGGATTTACTCTACGGCGATGTTGG
D-mel           ACTGGAGACTACACACACTACCACCAGCAGCTGGAGCACTTCCCCATCGAGCGGTGTCTG
A-nid           CCGGGTGACATGACTCATTTCAACCAAGAACTTAAGGACTGGCATGTCCCGTTGATGTAC
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                                                                    3180
B-tau           GATGAGTGCCTGAAGAGCTCTGAGTATTACGCTCGGAAGAGTGAGGTCGACAAGTTCAAC
H-sap           GAAGAATGCCTAGCAAGCTCTCAGTATCATGCTCGGAAGAGTGAGGTTGACAAGTTCAAC
G-gal           GATGAGTGTTTGTCAAGCTCTAACTATCATGCCAGGAAGAAACTAATTGAAGAATTCAAC
D-mel           GAGGATTGCTTGAAGCAGTCGAGATACGACGAGAAGCGGCAGGATATTGCTCGATTCAAT
A-nid           GATCAGGTTCTACAGGAGAGCGAGTATTTTGAGCGCCGCAAGGCCGTGGAGGAATATAAC

                                                                    3240
B-tau           AAGGAGAATTGTTGGAAAAAGAGAGGATTGTGCATAATTCCTACCAAATTTGGAATAAGC
H-sap           AAGGAGAATTGTTGGAAAAAGAGAGGATTGTGCATAATTCCCACCAAGTTTGGAATAAGC
G-gal           AAACAGAATCGCTGGAAGAAAAGAGGGATGTGTATTATTCCTACCAAATTTGGCATCAGC
D-mel           CGGGAGAATCGCTGGCGGAAACGCGGCATGGCGGTGGTGCCCACCAAGTATGGAATCGCA
A-nid           CGCACGCACAAGTGGTCCAAGCGTGGCATGGCCATTATCCCCACCAAGTTTGGTATCTCT

                                                                    3300
B-tau           TTCACAGTTCCTTTTCTAAATCAGGCAGGAGCCCTGATCCACGTATACACAGATGGCTCC
H-sap           TTCACAGTTCCTTTTCTGAATCAGGCAGGAGCCCTACTTCATGTGTACACAGATGGCTCT
G-gal           TTCACTGTCCCGTTTCTGAATCAGGCTGGAGCTCTGGTCCATGTATATACAGATGGCTCT
D-mel           TTTGGAGTGATGCACTTGAACCAAGCGGGATCGCTGATCAACATCTATGGTGATGGATCC
A-nid           TTCACGGCCCTCTTTCTCAACCAAGCGGGCGCCCTCGTTCATATCTACCACGACGGAAGC

                                                                    3360
B-tau           GTGCTGGTGAGCCACGGGGGCACAGAGATGG---AAGGCCTTCACACCAAGATGGTGCAG
H-sap           GTGCTGCTGACCCACGGGGGGACTGAGATGGGCCAAGGCCTTCATACCAAAATGGTCCAG
G-gal           GTATTACTTACACATGGTGGGACTGAGATGGGTCAAGGACTTCACACCAAAATGATCCAG
D-mel           GTGTTGCTTTCGCACGGAGGAGTTGAGATCGGACAAGGTCTGAATACCAAGATGATTCAG
A-nid           GTCCTCGTCGCCCACGGCGGCGTGGAAATGGGCCAAGGTCTCCACACAAAGATGACCATG

                                                                    3420
B-tau           GTGGCCAGCAAAGCTCTGAAGATCCCCATCTCTAAGATCTATATCAGCGAGACGAGCACT
H-sap           GTGGCCAGTAGAGCTCTGAAAATCCCCACCTCTAAGATTTATATCAGCGAGACAAGCACT
G-gal           GTTGCTAGCAGGTCACTGGGAATCCCTACCTCCAAAATTTACATCAGTGAGACCAGCACA
D-mel           TGCGCCGCCAGGGCTCTGGGGATTCCTTCGGAACTGATTCACATTTCGGAAACGGCCACG
A-nid           ATAGCAGCCGAAGCCCTAGGCGTTCCCTTGTCGGACGTCTTCATCTCCGAAACAGCCACC

                                                                    3480
B-tau           AACACGGTGCCCAACTCCTCTCCCACGGCCGCCTCCGTCAGTACCGACATCTATGGACAG
H-sap           AACACTGTGCCCAACACCTCTCCCACGGCTGCCTCTGTCAGCGCTGACCTCAATGGACAG
G-gal           AACACTGTCCCTAATACCTCCCCAACAGCTGCATCTGTCAGTGCTGACATCAATGGGATG
D-mel           GATAAAGTACCCAACACTTCACCCACGGCGGCGAGTGTGGGATCCGATCTGAACGGAATG
A-nid           AACACCGTCGCAAACACATCCTCTACAGCGGCTTCTGCCAGCTCCGACCTCAACGGCTAT

                                                                    3540
B-tau           GCCGTCTATGAAGCTTGCCAGACCATCCTGAAAAGGTTGGAACCCTTCAAGAAAAAGAAT
H-sap           GCCGTCTATGCGGCTTGTCAGACCATCTTGAAAAGGCTGGAACCCTACAAGAAGAAGAAT
G-gal           GCTGTTCATAATGCATGTCAGACCATCTTAAAAAGACTTGAGCCAATCAAACAGTCTAAT
D-mel           GCCGTACTGGATGCGTGTGAAAAGTTGAACAAAAGACTGGCGCCCATCAAGGAGGCATTG
A-nid           GCCATCTATAACGCCTGCACCCAACTGAACGAACGTCTCAAGCCCTACCGTGAAAAGATG

                                                                    3600
B-tau           CCTGATGGCTCCTGGGAAGACTGGGTCATGGCTGCCTACCAGGATAGAGTGAGCTTATCT
H-sap           CCCAGTGGCTCCTGGGAAGACTGGGTCACAGCTGCCTACATGGACACAGTGAGCTTGTCT
G-gal           CTCAAGGGGTCCTGGGAAGACTGGATTAAAACCGCCTATGAGAACTGCATTAGTCTGTCA
D-mel           CCTGGAGGCACCTGGAAGGAGTGGATCAACAAGGCGTATTTCGATCGGGTCAGCCTCTCG
A-nid           CCCAACGCAACCCTGAAAGACCTCGCCCACGCTGCTTACTTCGACCGTGTCAACCTTTCT

                                                                    3660
B-tau           ACCACTGGGTTTTACAGGACGCCCAACCTTGGCTACAGCTTTGAGACCAACTCAGGGAAT
H-sap           GCCACTGGGTTTTATAGAACACCCAATCTGGGCTACAGCTTTGAGACTAACTCAGGGAAC
G-gal           GCCACAGGATTTTATAGAATTCCTGACGTTGGCTACAACTTTGAAACGAACAAAGGGAAG
D-mel           GCCACAGGATTCTATGCCATGCCCGGGATTGGATATCACCCGGAAACGAATCCCAATGCT
A-nid           GCCCAAGGCTACTACCGCACCCCAGACATCGGCTATACCTGGGGTGAGAACAAGGGTCAA
```

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                                                                    3720
B-tau           GCCTTCCA---CTATTTCACCTACGGGGTGGCTTGCTCAGAAGTAGAAATTGACTGTTTG
H-sap           CGCTTCCA---CTACTTCAGCTATGGGGTGGCTTGCTCTGAAGTAGAAATCGACTGCCTA
G-gal           CCATTCCA---TTACTTCAGTTATGGTGTTGCCTGCTCCGAGGTTGAGATAGATTGCCTG
D-mel           CGCACCTATAGCTACTACACGAATGGCGTGGGAGTCACTGTGGTAGAGATCGATTGCCTG
A-nid           ATGTTCTT---CTACTTCACGCAGGGCGTTACAGCCGCTGAAGTCGAAATCGATACGCTC

                                        <----3745r------------
                                                                    3780
B-tau           ACAGGGGATCATAAGAACCTCCGTACAGACATTGTCATGGATGTTGGCTCCAGTCTGAAC
H-sap           ACAGGAGATCATAAGAACCTCCGCACAGATATTGTCATGGATGTTGGCTCCAGTCTAAAC
G-gal           ACAGGTGACCACAAGAACATCCGCACAGACATTGTTATGGATGTTGGCACCAGTCTGAAC
D-mel           ACTGGCGACCATCAGGTGCTCAGCACAGACATCGTGATGGACATCGGCTCTAGCCTGAAT
A-nid           ACCGGCGACTGGACGCCCCTCCGGGCAGATATCAAAATGGATGTCGGCCGCACAATCAAC
                                          ------------3768f----->

                                                                    3840
B-tau           CCTGCCATCGATATTGGACAGGTGGAAGGGGCGTTTGTCCAGGGCCTGGGCCTCTTCACC
H-sap           CCTGCCATTGATATTGGACAGGTGGAAGGGGCATTTGTCCAGGGCCTTGGCCTCTTCACC
G-gal           CCAGCCATAGATATAGGGCAGATAGAAGGGGCGTTTGTCCAAGGCATTGGGCTCTTCACC
D-mel           CCGGCTATTGACATTGGTCAGATCGAGGGAGCATTCATGCAGGGCTATGGACTGTTCACT
A-nid           CCCTCCATCGACTACGGTCAGATAGAAGGCGCATACATTCAGGGCCAGGGTCTCTTCACA

                                                                    3900
B-tau           CTGGAGGAG---CTACACTATTCCCCTGAGGGGAGCCTGCACACCCGCGGCCCCAGCACC
H-sap           CTAGAGGAG---CTACACTATTCCCCCGAGGGGAGCCTGCACACCCGTGGCCCTAGCACC
G-gal           ATGGAGGAG---TTGCGCTACTCACCTGAGGGGAACTTGTACACGCGAGGGCCTGGGATG
D-mel           TTGGAGGAA---CTCATGTACTCACCACAAGGCATGCTTTACTCCAGAGGTCCGGGCATG
A-nid           ACAGAAGAAAGCCTTTGGCACCGCACAACTGGCCAAATATTCACTAAAGGTCCTGGAAAC

                                                                    3960
B-tau           TACAAGATCCCCGCGTTTGGCAGCATCCCCACAGAGTTCAGGGTGTCCCTCGTTCGCGAC
H-sap           TACAAGATCCCGGCATTTGGCAGCATCCCCATTGAGTTCAGGGTGTCCCTGCTCCGCGAC
G-gal           TACAAAATCCCAGCATTTGGAGACATCCCAACAGAATTTTACGTGTCTCTTCTCCGTGAC
D-mel           TACAAGCTGCCAGGATTTGCCGACATTCCCGGGGAGTTCAATGTCAGCCTACTGACCGGT
A-nid           TACAAAATTCCGGGCTTCCGTGACATTCCGCAAATATTCAACGTTAGTCTCCTTAAGGAC

                                                  <----4003r-------
                                                                    4020
B-tau           ------TGCCCCAACAAGAAGGCCATCTATGCCTCCAAGGCGGTCGGGGAGCCGCCCCTC
H-sap           ------TGCCCCAACAAGAAGGCCATCTATGCATCGAAGGCTGTTGGAGAGCCGCCCCTC
G-gal           ------TGCCCCAACAGCAAGGCAATTTATTCATCCAAGGCTGTGGGAGAGCCGCCTCTG
D-mel           ------GCCCCCAATCCACGGGCAGTCTACTCTTCCAAGGCAGTGGGTGAACCTCCGCTC
A-nid           GTAGAGTGGGAGAACCTGCGGACGATCCAGCGCAGTAGAGGCGTCGGCGAGCCACCGCTT

                --
                                                                    4080
B-tau           TTCCTGGGGCCCTCCGTCTTCTTTGCCATCAAGGATGCCATCGCGCGCGG-TCGAGCTCA
H-sap           TTCCTGGCTGCTTCTATCTTCTTTGCCATCAAAGATGCCATC-CGTGCAGCTCGAGCTCA
G-gal           TTCCTGTCTGCTTCAGTGTTTTATGCCATCAAAGATGCTATC-TACTCAG--------CA
D-mel           TTCATTGGATCATCTGCATTCTTTGCCATTAAGGAGGCCATT----GCAG------CTGC
A-nid           TTCATGGGCAGCGCGGCGTTTTTCGCTATTCGGGATGC-ACT----GAAG-----GCTGC
                                                                    4140
B-tau           GCACACAAATAATAACACGAAGGAGCTCTTCCGGCTAGATAGCCCTGCCACCCCGGAGAA
H-sap           GCACACAGGTAATAACGTGAAGGAACTCTTCCGCCTAGACAGCCCTGCCACCCCGGAGAA
G-gal           AGGGA-AGACTCTGGCGTGACGGAACCATTCAGGCTGGACAGCCCTGCCACCCCAGAGAG
D-mel           TCGCGAGGATCAGGGCTTGAGTGGTGACTTCCCACTGGAGGCGCCTTCCACATCGGCACG
A-nid           CAGGAAGGAGTGGGGGGTTACTGATGTGCTGAGTCTTGTCAGTCCGGCTACACCGGAGAG

                                                                    4200
B-tau           GATCCGCAATGCCTGCGTGGACAAGTTCACCACTCTGTGTGTCACTGGTGCACCAGGAAA
H-sap           GATCCGCAATGCCTGCGTGGACAAGTTCACCACCCTGTGTGTCACTGGTGTCCCAGAAAA
G-gal           GATCCGCAATGCCTGTGTGGACACCTTTACAAAAATGTGCCCTTCTGCTGAGCCAGGGAC
D-mel           CATTCGAATTGCTTGTCAGGATAAGTTCACGGAACTGCTTGAAATACCCGAACCAGGATC
A-nid           AATTAGGGTTAGTTGTGCGGATCCCATTATTGAAAGGGCGAGAGTGAAGGCGGAGGAAGG
```

```
                                        4228
B-tau           CTGTAAACCCTGGTCTCTGAGGGTCTGA
H-sap           CTGCAAACCCTGGTCTGTGAGGGTC---
G-gal           CTTTAAGCCATGGTCTGTGCGTGCGTAA
D-mel           ATTTACGCCATGGAACAT-TGTGCCT--
A-nid           GGAAAAGAGCTTCTTCGTTGCGATATAG
```

# APPENDIX 3

Retention factors of phenolic compounds from elicited and control cassava cells. See Chapter 5, **Figure 5.8**.

| Rf* (elicited cells) | Time (hours) | | | | | | 254 nm | 366 nm |
|---|---|---|---|---|---|---|---|---|
| | 0 | 6 | 12 | 24 | 48 | 72 | | |
| 0.0 | + | + | + | + | + | + | v | v |
| 0.053 | + | + | + | + | + | + | nv | v |
| 0.17 | + | + | + | + | + | + | nv | v |
| 0.18 | + | + | + | + | + | + | nv | v |
| 0.214 | - | - | - | - | + | + | v | v |
| 0.268 | + | + | + | + | + | + | nv | v |
| 0.321 | + | + | + | + | + | + | v | v |
| 0.357 | + | + | + | + | + | + | nv | v |
| 0.464 | + | + | + | + | + | + | nv | v |
| 0.535 | - | - | - | - | + | + | nv | v |
| 0.607 | + | + | + | + | + | + | v | v |
| 0.642 | - | - | - | - | + | + | v | v |
| 0.72 | - | - | - | - | + | + | v | v |
| 0.767 | + | + | + | + | + | + | v | v |
| 0.84 | - | - | - | - | + | + | nv | v |

**Rf**: retention factor**+**: band present; **-**: band absent; **v**: band visible under UV; **nv**: band not visible under UV.

| Rf* (control cells) | Time (hours) | | | | | | 254 nm | 366 nm |
|---|---|---|---|---|---|---|---|---|
| | 0 | 6 | 12 | 24 | 48 | 72 | | |
| 0.0 | + | + | + | + | + | + | v | v |
| 0.053 | + | + | + | + | + | + | nv | v |
| 0.17 | + | + | + | + | + | + | nv | v |
| 0.18 | + | + | + | + | + | + | nv | v |
| 0.214 | - | - | - | - | + | + | v | v |
| 0.268 | + | + | + | + | + | + | nv | v |
| 0.321 | + | + | + | + | + | + | v | v |
| 0.357 | + | + | + | + | + | + | nv | v |
| 0.464 | + | + | + | + | + | + | nv | v |
| 0.535 | - | - | - | - | + | + | nv | v |
| 0.607 | + | + | + | + | + | + | v | v |
| 0.767 | + | + | + | + | + | + | v | v |
| 0.84 | - | - | - | - | + | + | nv | v |

**Rf**: retention factor**+**: band present; **-**: band absent; **v**: band visible under UV; **nv**: band not visible under UV.

# APPENDIX 4

Retention factors of terpenoids compounds from elicited and control cassava cells. See Chapter 5, **Figure 5.9**.

| Rf* (elicited cells) | Time (hours) | | | | | | 254 nm | 366 nm |
|---|---|---|---|---|---|---|---|---|
| | 0 | 6 | 12 | 24 | 48 | 72 | | |
| 0.0 | + | + | + | + | + | + | nv | v |
| 0.06 | + | + | + | + | + | + | nv | v |
| 0.1 | + | + | + | + | + | + | nv | v |
| 0.16 | - | - | - | - | + | + | nv | v |
| 0.18 | + | + | + | + | + | + | nv | nv |
| 0.22 | + | + | + | + | + | + | nv | v |
| 0.28 | + | + | + | + | + | + | v | v |
| 0.34 | + | + | + | + | + | + | v | v |
| 0.36 | + | + | + | + | + | + | nv | nv |
| 0.4 | + | + | + | + | + | + | nv | v |
| 0.48 | - | - | - | - | + | + | nv | v |
| 0.52 | + | + | + | + | + | + | nv | v |
| 0.58 | - | - | - | - | + | + | nv | v |
| 0.62 | + | + | + | + | + | + | nv | nv |
| 0.68 | + | + | + | + | + | + | v | v |
| 0.76 | + | + | + | + | + | + | nv | v |
| 0.82 | + | + | + | + | + | + | v | v |

**Rf**: retention factor**+**: band present; **-**: band absent; **v**: band visible under UV; **nv**: band not visible under UV.

| Rf* (control cells) | Time (hours) | | | | | | 254 nm | 366 nm |
|---|---|---|---|---|---|---|---|---|
| | 0 | 6 | 12 | 24 | 48 | 72 | | |
| 0.0 | + | + | + | + | + | + | v | v |
| 0.06 | + | + | + | + | + | + | v | v |
| 0.1 | - | - | - | + | + | + | nv | v |
| 0.16 | - | - | - | + | + | + | nv | v |
| 0.22 | + | + | + | + | + | + | nv | v |
| 0.28 | + | + | + | + | + | + | nv | v |
| 0.34 | + | + | + | + | + | + | v | v |
| 0.36 | + | + | + | + | + | + | nv | nv |
| 0.4 | + | + | + | + | + | + | nv | nv |
| 0.44 | - | - | - | - | + | + | nv | v |
| 0.48 | + | + | + | + | + | - | nv | nv |
| 0.52 | + | + | + | + | + | + | nv | nv |
| 0.62 | + | + | - | + | + | + | nv | v |
| 0.70 | + | + | + | + | + | + | v | nv |
| 0.76 | - | - | - | - | + | + | nv | v |
| 0.82 | + | + | + | + | + | + | v | v |

**Rf**: retention factor**+**: band present; **-**: band absent; **v**: band visible under UV; **nv**: band not visible under UV.

# APPENDIX 5

Retention factors of the bands in the TLC bioassay with cassava cell extracts and *T. harzianum*. See Chapter 5, **Figure 5.24.**

| Rf* | Control cells (extracted with pH 5.0) | Control cells (extracted with pH 8.0) | Elicited[A] cells (extracted with pH 5.0) | Elicited[B] cells (extracted with pH 8.0) | Elicited[C] cells (extracted with pH 5.0) | Elicited[D] cells (extracted with pH 8.0) |
|---|---|---|---|---|---|---|
| | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 0.038 | 0.053 | 0.053 | 0.053 | 0.053 | 0.053 |
| | 0.053 | 0.1846 | 0.107 | 0.1076 | 0.1076 | 0.5 |
| | 0.192 | 0.438 | 0.492 | 0.48 | 0.192 | 0.692 |
| | 0.495 | 0.646 | 0.646 | 0.653 | 0.5 | |
| | 0.692 | | | | 0.692 | |
| | | | | | 0.73 | |

**A**: Elicited cells (50 μg/ml); **B**: Elicited cells (50 μg/ml); **C**: Elicited cells (100 μg/ml); **D**: Elicited cells (100 μg/ml). Note that cells extracts at only 48 h following elicitation.

# APPENDIX 6

Retention factors of the bands in the TLC bioassay with cassava cell extracts and *F. avenae*. See Chapter 5, **Figure 5.25**.

| Rf* | Control cells | Elicited cells (50 μg/ml) | Elicited cells (100 μg/ml) |
|---|---|---|---|
| | 0.0 | 0.0 | 0.0 |
| | 0.177 | 0.177 | 0.177 |
| | 0.205 | 0.205 | 0.205 |
| | 0.24 | 0.24 | 0.24 |
| | 0.27 | 0.27 | 0.27 |
| | 0.52 | 0.52 | 0.52 |
| | 0.70 | 0.70 | 0.70 |
| | 0.75 | - | - |
| | 0.83 | 0.83 | 0.83 |
| | 0.89 | 0.89 | 0.89 |

Note that cells extracts at 48 h only.

# APPENDIX 7

Analysis of variance (ANOVA) tables. See Chapter 5. Slide germination assay. One way analysis of variance applied for all the tables.

*T. harzianum*
ESCULETIN
GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 22391.5 | 3731.9 | 89.98 | 0.000 |
| Error | 35 | 1451.7 | 41.5 | | |
| Total | 41 | 23843.1 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 79.333 | 5.428 |
| 10 | 6 | 67.000 | 7.376 |
| 50 | 6 | 57.000 | 5.865 |
| 100 | 6 | 46.167 | 7.055 |
| 200 | 6 | 33.667 | 5.750 |
| 500 | 6 | 20.167 | 6.014 |
| 1000 | 6 | 10.667 | 7.285 |

GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 1305.3 | 217.5 | 17.59 | 0.000 |
| Error | 35 | 432.8 | 12.4 | | |
| Total | 41 | 1738.1 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 40.000 | 2.757 |
| 10 | 6 | 40.000 | 3.847 |
| 50 | 6 | 40.000 | 3.847 |
| 100 | 6 | 37.000 | 2.608 |
| 200 | 6 | 29.667 | 3.204 |
| 500 | 6 | 29.500 | 4.231 |
| 1000 | 6 | 26.000 | 3.795 |

FERULIC ACID
GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 35695.3 | 5949.2 | 193.37 | 0.000 |
| Error | 35 | 1076.8 | 30.8 | | |
| Total | 41 | 36772.1 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 79.333 | 5.428 |
| 10 | 6 | 67.667 | 7.174 |
| 50 | 6 | 53.833 | 7.960 |
| 100 | 6 | 34.667 | 4.179 |
| 200 | 6 | 18.667 | 6.055 |
| 500 | 6 | 1.667 | 4.082 |
| 1000 | 6 | 0.333 | 0.516 |

## GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 7570.2 | 1261.7 | 108.19 | 0.000 |
| Error | 35 | 408.2 | 11.7 | | |
| Total | 41 | 7978.4 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 40.000 | 2.757 |
| 10 | 6 | 40.000 | 3.847 |
| 50 | 6 | 39.333 | 2.658 |
| 100 | 6 | 19.333 | 4.367 |
| 200 | 6 | 16.000 | 3.406 |
| 500 | 6 | 13.500 | 3.619 |
| 1000 | 6 | 6.000 | 2.898 |

## QUERCETIN GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 6111.9 | 1018.7 | 30.62 | 0.000 |
| Error | 35 | 1164.5 | 33.3 | | |
| Total | 41 | 7276.4 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 79.333 | 5.428 |
| 10 | 6 | 78.667 | 4.885 |
| 50 | 6 | 65.833 | 7.414 |
| 100 | 6 | 55.333 | 6.121 |
| 200 | 6 | 55.333 | 4.761 |
| 500 | 6 | 52.167 | 5.565 |
| 1000 | 6 | 46.500 | 5.788 |

## GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 2784.2 | 464.0 | 40.84 | 0.000 |
| Error | 35 | 397.7 | 11.4 | | |
| Total | 41 | 3181.9 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 40.000 | 3.899 |
| 10 | 6 | 22.000 | 4.147 |
| 50 | 6 | 20.000 | 3.899 |
| 100 | 6 | 18.000 | 2.828 |
| 200 | 6 | 16.833 | 2.858 |
| 500 | 6 | 15.500 | 2.881 |
| 1000 | 6 | 14.333 | 2.733 |

## SCOPOLETIN GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 29563.9 | 4927.3 | 161.78 | 0.000 |
| Error | 35 | 1066.0 | 30.5 | | |
| Total | 41 | 30629.9 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 79.333 | 5.428 |
| 10 | 6 | 70.667 | 8.042 |
| 50 | 6 | 68.833 | 4.355 |
| 100 | 6 | 56.833 | 5.742 |
| 200 | 6 | 47.333 | 5.317 |
| 500 | 6 | 15.833 | 4.491 |
| 1000 | 6 | 4.500 | 4.324 |

GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 5555.81 | 925.97 | 131.12 | 0.000 |
| Error | 35 | 247.17 | 7.06 | | |
| Total | 41 | 5802.98 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 40.000 | 3.847 |
| 10 | 6 | 22.667 | 3.386 |
| 50 | 6 | 16.000 | 1.897 |
| 100 | 6 | 12.833 | 2.137 |
| 200 | 6 | 12.333 | 2.160 |
| 500 | 6 | 5.167 | 2.483 |
| 1000 | 6 | 3.167 | 2.041 |

*F. solani*

ESCULETIN

GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 9157.3 | 1526.2 | 89.80 | 0.000 |
| Error | 35 | 594.8 | 17.0 | | |
| Total | 41 | 97.52.1 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 99.50 | 0.84 |
| 10 | 6 | 99.50 | 0.84 |
| 50 | 6 | 94.33 | 3.27 |
| 100 | 6 | 89.33 | 3.44 |
| 200 | 6 | 74.33 | 5.38 |
| 500 | 6 | 66.50 | 5.43 |
| 1000 | 6 | 61.17 | 6.05 |

GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 307689 | 51282 | 66.83 | 0.000 |
| Error | 35 | 26858 | 767 | | |
| Total | 41 | 334548 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 345.00 | 28.81 |
| 10 | 6 | 305.83 | 15.30 |
| 50 | 6 | 248.33 | 33.42 |
| 100 | 6 | 235.00 | 29.66 |
| 200 | 6 | 193.33 | 19.41 |
| 500 | 6 | 117.50 | 17.82 |
| 1000 | 6 | 93.33 | 40.21 |

FERULIC ACID

GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 33922.62 | 5653.77 | 885.38 | 0.000 |
| Error | 35 | 223.50 | 6.39 | | |
| Total | 41 | 34146.12 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 99.50 | 0.84 |
| 10 | 6 | 99.50 | 0.84 |
| 50 | 6 | 99.33 | 0.82 |
| 100 | 6 | 96.17 | 2.48 |
| 200 | 6 | 90.50 | 3.62 |
| 500 | 6 | 67.00 | 2.97 |
| 1000 | 6 | 16.83 | 3.82 |

## GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 609637 | 101606 | 273.82 | 0.000 |
| Error | 35 | 12987 | 371 | | |
| Total | 41 | 622624 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 345.00 | 28.81 |
| 10 | 6 | 336.67 | 22.95 |
| 50 | 6 | 276.67 | 17.80 |
| 100 | 6 | 184.17 | 21.08 |
| 200 | 6 | 98.33 | 19.15 |
| 500 | 6 | 71.67 | 8.16 |
| 1000 | 6 | 28.33 | 6.83 |

## QUERCETIN GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 444.57 | 74.10 | 9.20 | 0.000 |
| Error | 35 | 281.83 | 8.05 | | |
| Total | 41 | 726.40 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 99.50 | 0.84 |
| 10 | 6 | 99.50 | 0.84 |
| 50 | 6 | 99.17 | 0.98 |
| 100 | 6 | 99.00 | 1.26 |
| 200 | 6 | 98.67 | 1.51 |
| 500 | 6 | 92.33 | 6.65 |
| 1000 | 6 | 91.67 | 2.42 |

## GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 579987 | 96664 | 168.11 | 0.000 |
| Error | 35 | 20125 | 575 | | |
| Total | 41 | 600112 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 345.00 | 28.81 |
| 10 | 6 | 335.00 | 21.91 |
| 50 | 6 | 323.33 | 26.77 |
| 100 | 6 | 235.00 | 19.49 |
| 200 | 6 | 99.17 | 17.44 |
| 500 | 6 | 89.17 | 16.56 |
| 1000 | 6 | 60.00 | 32.25 |

## SCOPOLETIN GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 377.48 | 62.91 | 20.08 | 0.000 |
| Error | 35 | 109.67 | 3.13 | | |
| Total | 41 | 487.14 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 99.50 | 0.84 |
| 10 | 6 | 99.50 | 0.55 |
| 50 | 6 | 99.50 | 0.84 |
| 100 | 6 | 99.50 | 0.84 |
| 200 | 6 | 99.50 | 0.84 |
| 500 | 6 | 98.67 | 1.97 |
| 1000 | 6 | 90.37 | 3.87 |

GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 481045 | 80174 | 218.73 | 0.000 |
| Error | 35 | 12829 | 367 | | |
| Total | 41 | 493874 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 345.00 | 28.81 |
| 10 | 6 | 335.83 | 23.11 |
| 50 | 6 | 261.67 | 18.07 |
| 100 | 6 | 242.50 | 19.69 |
| 200 | 6 | 101.67 | 12.52 |
| 500 | 6 | 90.83 | 12.01 |
| 1000 | 6 | 81.67 | 13.66 |

*F. oxysporum*
ESCULETIN
GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 3082.6 | 513.8 | 26.71 | 0.000 |
| Error | 35 | 673.3 | 19.2 | | |
| Total | 41 | 3755.9 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 98.83 | 1.17 |
| 10 | 6 | 97.50 | 2.51 |
| 50 | 6 | 96.33 | 2.16 |
| 100 | 6 | 90.83 | 5.31 |
| 200 | 6 | 87.00 | 5.22 |
| 500 | 6 | 84.00 | 5.22 |
| 1000 | 6 | 72.83 | 6.31 |

GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 106521 | 17753 | 0.49 | 0.000 |
| Error | 35 | 1276741 | 36478 | | |
| Total | 41 | 1383262 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 450.8 | 189.2 |
| 10 | 6 | 434.2 | 20.54 |
| 50 | 6 | 416.7 | 20.53 |
| 100 | 6 | 384.2 | 20.69 |
| 200 | 6 | 369.2 | 20.27 |
| 500 | 6 | 311.3 | 14.90 |
| 1000 | 6 | 319.2 | 17.07 |

FERULIC ACID
GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 7985.5 | 1330.9 | 62.82 | 0.000 |
| Error | 35 | 741.5 | 21.2 | | |
| Total | 41 | 8727.0 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 98.83 | 1.17 |
| 10 | 6 | 98.67 | 1.51 |
| 50 | 6 | 91.50 | 5.96 |
| 100 | 6 | 87.50 | 7.50 |
| 200 | 6 | 85.00 | 3.58 |
| 500 | 6 | 69.50 | 3.45 |
| 1000 | 6 | 59.17 | 5.31 |

## GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 167131 | 27855 | 0.41 | 0.000 |
| Error | 35 | 2376539 | 67901 | | |
| Total | 41 | 2543670 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 402.5 | 273.4 |
| 10 | 6 | 381.3 | 281.6 |
| 50 | 6 | 358.0 | 284.4 |
| 100 | 6 | 316.0 | 281.7 |
| 200 | 6 | 284.8 | 268.2 |
| 500 | 6 | 235.5 | 210.5 |
| 1000 | 6 | 233.0 | 211.8 |

## QUERCETIN GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 6667.6 | 1111.3 | 53.32 | 0.000 |
| Error | 35 | 729.5 | 20.8 | | |
| Total | 41 | 7397.1 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 98.83 | 1.17 |
| 10 | 6 | 97.50 | 2.17 |
| 50 | 6 | 89.83 | 6.01 |
| 100 | 6 | 88.83 | 4.17 |
| 200 | 6 | 77.67 | 4.13 |
| 500 | 6 | 70.67 | 7.09 |
| 1000 | 6 | 63.17 | 4.36 |

## GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 602173 | 100362 | 49.38 | 0.000 |
| Error | 35 | 71142 | 2033 | | |
| Total | 41 | 673314 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 645.00 | 62.61 |
| 10 | 6 | 643.33 | 50.56 |
| 50 | 6 | 521.67 | 39.20 |
| 100 | 6 | 450.83 | 53.05 |
| 200 | 6 | 390.83 | 39.30 |
| 500 | 6 | 378.33 | 32.51 |
| 1000 | 6 | 320.0 | 28.28 |

## SCOPOLETIN GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 18.33 | 3.06 | 0.58 | 0.000 |
| Error | 35 | 183.67 | 5.25 | | |
| Total | 41 | 202.0 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 98.83 | 1.17 |
| 10 | 6 | 98.83 | 1.17 |
| 50 | 6 | 98.50 | 2.35 |
| 100 | 6 | 97.83 | 1.94 |
| 200 | 6 | 97.50 | 2.95 |
| 500 | 6 | 97.33 | 2.66 |
| 1000 | 6 | 97.17 | 2.99 |

GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 6 | 1067974 | 177996 | 213.59 | 0.000 |
| Error | 35 | 29167 | 833 | | |
| Total | 41 | 1097140 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0 | 6 | 645.00 | 62.61 |
| 10 | 6 | 413.33 | 16.02 |
| 50 | 6 | 344.17 | 15.63 |
| 100 | 6 | 293.33 | 24.83 |
| 200 | 6 | 250.83 | 18.82 |
| 500 | 6 | 164.17 | 17.72 |
| 1000 | 6 | 137.50 | 11.29 |

Analysis of variance (ANOVA) Tables for oxidation assay

*T. harzianum*

GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 12 | 89370.46 | 7447.54 | 1113.28 | 0.00 |
| Error | 65 | 434.83 | 6.69 | | |
| Total | 77 | 89805.29 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 1 | 6 | 0.00 | 0.00 |
| 2 | 6 | 50.33 | 2.25 |
| 3 | 6 | 69.00 | 3.35 |
| 4 | 6 | 23.83 | 2.40 |
| 5 | 6 | 37.00 | 2.53 |
| 6 | 6 | 65.67 | 3.93 |
| 7 | 6 | 27.00 | 1.41 |
| 8 | 6 | 98.50 | 3.21 |
| 9 | 6 | 98.17 | 2.23 |
| 10 | 6 | 97.83 | 2.71 |
| 11 | 6 | 97.67 | 2.73 |
| 12 | 6 | 97.33 | 2.16 |
| 13 | 6 | 98.50 | 2.51 |

GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 12 | 46567.2 | 3880.6 | 306.98 | 0.00 |
| Error | 65 | 821.7 | 12.6 | | |
| Total | 77 | 47388.9 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 1 | 6 | 0.00 | 0.00 |
| 2 | 6 | 14.167 | 1.722 |
| 3 | 6 | 28.00 | 1.414 |
| 4 | 6 | 13.500 | 1.225 |
| 5 | 6 | 27.500 | 3.209 |
| 6 | 6 | 29.000 | 3.521 |
| 7 | 6 | 12.667 | 1.211 |
| 8 | 6 | 63.833 | 5.456 |
| 9 | 6 | 63.833 | 2.483 |
| 10 | 6 | 64.833 | 3.545 |
| 11 | 6 | 65.000 | 3.406 |
| 12 | 6 | 65.000 | 4.733 |
| 13 | 6 | 65.000 | 7.155 |

*F. solani*

GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 12 | 27017.18 | 2251.43 | 239.32 | 0.00 |
| Error | 65 | 611.50 | 9.41 | | |
| Total | 77 | 27628.68 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 1 | 6 | 52.17 | 4.58 |
| 2 | 6 | 58.00 | 4.24 |
| 3 | 6 | 95.50 | 4.18 |
| 4 | 6 | 54.17 | 2.93 |
| 5 | 6 | 90.17 | 2.93 |
| 6 | 6 | 91.17 | 2.40 |
| 7 | 6 | 66.83 | 5.49 |
| 8 | 6 | 99.17 | 2.04 |
| 9 | 6 | 98.50 | 2.35 |
| 10 | 6 | 98.50 | 1.76 |
| 11 | 6 | 100.00 | 0.00 |
| 12 | 6 | 100.00 | 0.00 |
| 13 | 6 | 100.00 | 0.00 |

GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 12 | 183008 | 15251 | 32.37 | 0.00 |
| Error | 65 | 30625 | 471 | | |
| Total | 77 | 213633 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 1 | 6 | 250.0 | 18.17 |
| 2 | 6 | 244.17 | 19.34 |
| 3 | 6 | 268.33 | 23.80 |
| 4 | 6 | 139.17 | 18.28 |
| 5 | 6 | 197.50 | 16.36 |
| 6 | 6 | 250.83 | 22.23 |
| 7 | 6 | 235.83 | 11.58 |
| 8 | 6 | 300.83 | 33.38 |
| 9 | 6 | 305.00 | 31.30 |
| 10 | 6 | 300.00 | 25.50 |
| 11 | 6 | 305.00 | 18.71 |
| 12 | 6 | 300.00 | 8.37 |
| 13 | 6 | 305.00 | 21.45 |

*F. oxysporum*

GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 12 | 32389.9 | 2699.2 | 124.65 | 0.00 |
| Error | 65 | 1407.5 | 21.7 | | |
| Total | 77 | 33797.4 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 1 | 6 | 51.83 | 4.26 |
| 2 | 6 | 45.67 | 8.19 |
| 3 | 6 | 61.50 | 5.61 |
| 4 | 6 | 57.33 | 4.76 |
| 5 | 6 | 73.33 | 6.02 |
| 6 | 6 | 65.33 | 7.23 |
| 7 | 6 | 70.00 | 6.32 |
| 8 | 6 | 98.50 | 2.51 |
| 9 | 6 | 98.50 | 2.35 |
| 10 | 6 | 99.00 | 1.26 |
| 11 | 6 | 100.00 | 0.00 |
| 12 | 6 | 99.83 | 0.41 |
| 13 | 6 | 100.00 | 0.00 |

GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 12 | 64662 | 5389 | 4.39 | 0.00 |
| Error | 65 | 79775 | 1227 | | |
| Total | 77 | 1444437 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 1 | 6 | 342.50 | 33.43 |
| 2 | 6 | 356.67 | 25.43 |
| 3 | 6 | 392.50 | 18.64 |
| 4 | 6 | 384.17 | 17.72 |
| 5 | 6 | 385.83 | 26.72 |
| 6 | 6 | 395.83 | 14.97 |
| 7 | 6 | 337.50 | 25.45 |
| 8 | 6 | 416.67 | 36.01 |
| 9 | 6 | 415.83 | 56.69 |
| 10 | 6 | 418.33 | 45.02 |
| 11 | 6 | 417.50 | 43.67 |
| 12 | 6 | 419.17 | 43.87 |
| 13 | 6 | 419.17 | 39.68 |

Analysis of variance (ANOVA). Tables for assay of cells extracts.

*F. solani*

Control cells

GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 14 | 149103.2 | 10650.2 | 492.46 | 0.00 |
| Error | 75 | 1622.0 | 21.6 | | |
| Total | 89 | 150725.2 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0-0.05 | 6 | 99.50 | 1.22 |
| 0-0.5 | 6 | 17.17 | 5.53 |
| 0-5 | 6 | 0.00 | 0.00 |
| 12-0.05 | 6 | 98.83 | 1.17 |
| 12-0.5 | 6 | 28.83 | 8.52 |
| 12-5 | 6 | 0.00 | 0.00 |
| 24-0.05 | 6 | 99.50 | 0.84 |
| 24-0.5 | 6 | 77.00 | 8.27 |
| 24-5 | 6 | 0.00 | 0.00 |
| 48-0.05 | 6 | 99.17 | 1.33 |
| 48-0.5 | 6 | 95.17 | 4.92 |
| 48-5 | 6 | 49.33 | 7.76 |
| 72-0.05 | 6 | 99.33 | 1.21 |
| 72-0.5 | 6 | 80.17 | 4.26 |
| 72-5 | 6 | 20.33 | 6.59 |

Elicited cells

GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 14 | 156481.3 | 11177.2 | 679.42 | 0.00 |
| Error | 75 | 1233.8 | 16.5 | | |
| Total | 89 | 157715.1 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0-0.05 | 6 | 99.50 | 1.22 |
| 0-0.5 | 6 | 17.17 | 5.53 |
| 0-5 | 6 | 0.00 | 0.00 |
| 12-0.05 | 6 | 99.50 | 0.84 |
| 12-0.5 | 6 | 31.00 | 5.22 |
| 12-5 | 6 | 0.00 | 0.00 |
| 24-0.05 | 6 | 99.33 | 1.21 |
| 24-0.5 | 6 | 34.50 | 7.06 |
| 24-5 | 6 | 0.00 | 0.00 |
| 48-0.05 | 6 | 99.50 | 0.55 |
| 48-0.5 | 6 | 48.17 | 5.78 |
| 48-5 | 6 | 0.00 | 0.00 |
| 72-0.05 | 6 | 99.50 | 0.84 |
| 72-0.5 | 6 | 50.67 | 10.05 |
| 72-5 | 6 | 0.00 | 0.00 |

*F. solani*
Control cells
GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 14 | 2098204 | 149872 | 371.33 | 0.00 |
| Error | 75 | 30271 | 404 | | |
| Total | 89 | 2128475 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0-0.05 | 6 | 350 | 28.81 |
| 0-0.5 | 6 | 191.67 | 16.02 |
| 0-5 | 6 | 0.00 | 0.00 |
| 12-0.05 | 6 | 346.67 | 29.78 |
| 12-0.5 | 6 | 156.67 | 16.93 |
| 12-5 | 6 | 0.00 | 0.00 |
| 24-0.05 | 6 | 350.00 | 28.81 |
| 24-0.5 | 6 | 65.00 | 14.49 |
| 24-5 | 6 | 0.00 | 0.00 |
| 48-0.05 | 6 | 337.50 | 27.70 |
| 48-0.5 | 6 | 26.67 | 14.02 |
| 48-5 | 6 | 0.00 | 0.00 |
| 72-0.05 | 6 | 350.00 | 31.30 |
| 72-0.5 | 6 | 300.00 | 28.46 |
| 72-5 | 6 | 0.00 | 0.00 |

*F. solani*
Elicited cells
GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 14 | 1792459 | 128033 | 233.93 | 0.00 |
| Error | 75 | 41049 | 547 | | |
| Total | 89 | 1833508 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0-0.05 | 6 | 350 | 28.81 |
| 0-0.5 | 6 | 342.50 | 30.12 |
| 0-5 | 6 | 0.00 | 0.00 |
| 12-0.05 | 6 | 328.33 | 24.01 |
| 12-0.5 | 6 | 206.67 | 16.63 |
| 12-5 | 6 | 0.00 | 0.00 |
| 24-0.05 | 6 | 340.83 | 29.23 |
| 24-0.5 | 6 | 272.50 | 25.25 |
| 24-5 | 6 | 0.00 | 0.00 |
| 48-0.05 | 6 | 350.00 | 31.30 |
| 48-0.5 | 6 | 321.67 | 35.31 |
| 48-5 | 6 | 176.67 | 20.17 |
| 72-0.05 | 6 | 339.17 | 31.85 |
| 72-0.5 | 6 | 90.00 | 19.49 |
| 72-5 | 6 | 36.83 | 9.99 |

*T. harzianum*

Control cells

GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 14 | 142478.3 | 10177.0 | 249.25 | 0.00 |
| Error | 75 | 3062 | 40.8 | | |
| Total | 89 | 145540.6 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0-0.05 | 6 | 98.67 | 1.75 |
| 0-0.5 | 6 | 19.17 | 15.13 |
| 0-5 | 6 | 0.00 | 0.00 |
| 12-0.05 | 6 | 98.17 | 1.47 |
| 12-0.5 | 6 | 55.33 | 8.78 |
| 12-5 | 6 | 0.00 | 0.00 |
| 24-0.05 | 6 | 88.17 | 6.59 |
| 24-0.5 | 6 | 71.50 | 10.93 |
| 24-5 | 6 | 0.00 | 0.00 |
| 48-0.05 | 6 | 100.00 | 0.00 |
| 48-0.5 | 6 | 83.50 | 6.72 |
| 48-5 | 6 | 55.33 | 6.56 |
| 72-0.05 | 6 | 99.83 | 0.41 |
| 72-0.5 | 6 | 35.00 | 7.07 |
| 72-5 | 6 | 0.00 | 0.00 |

Elicited cells

GERMINATION

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 14 | 154752.2 | 11053.7 | 380.75 | 0.00 |
| Error | 75 | 2177.3 | 29.0 | | |
| Total | 89 | 156929.6 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0-0.05 | 6 | 98.67 | 1.75 |
| 0-0.5 | 6 | 19.17 | 15.13 |
| 0-5 | 6 | 0.00 | 0.00 |
| 12-0.05 | 6 | 100.00 | 0.00 |
| 12-0.5 | 6 | 58.17 | 6.85 |
| 12-5 | 6 | 0.00 | 0.00 |
| 24-0.05 | 6 | 99.50 | 1.22 |
| 24-0.5 | 6 | 49.00 | 7.38 |
| 24-5 | 6 | 0.00 | 0.00 |
| 48-0.05 | 6 | 100.00 | 0.00 |
| 48-0.5 | 6 | 50.33 | 7.00 |
| 48-5 | 6 | 0.00 | 0.00 |
| 72-0.05 | 6 | 100.00 | 0.00 |
| 72-0.5 | 6 | 48.50 | 7.18 |
| 72-5 | 6 | 0.00 | 0.00 |

*T. harzianum*

Control cells

GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 14 | 23877.01 | 1705.50 | 198.84 | 0.00 |
| Error | 75 | 643.31 | 8.58 | | |
| Total | 89 | 24520.32 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0-0.05 | 6 | 40.00 | 4.243 |
| 0-0.5 | 6 | 20.167 | 2.787 |
| 0-5 | 6 | 0.00 | 0.000 |
| 12-0.05 | 6 | 39.500 | 4.037 |
| 12-0.5 | 6 | 31.533 | 3.264 |
| 12-5 | 6 | 0.00 | 0.000 |
| 24-0.05 | 6 | 40.00 | 3.847 |
| 24-0.5 | 6 | 27.333 | 2.733 |
| 24-5 | 6 | 0.000 | 0.000 |
| 48-0.05 | 6 | 39.833 | 2.229 |
| 48-0.5 | 6 | 14.000 | 1.789 |
| 48-5 | 6 | 5.500 | 4.087 |
| 72-0.05 | 6 | 39.667 | 5.046 |
| 72-0.5 | 6 | 18.325 | 1.829 |

Elicited cells

GERM TUBE LENGTH

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Concentration | 14 | 23812.62 | 1700.90 | 279.45 | 0.00 |
| Error | 75 | 456.50 | 6.09 | | |
| Total | 89 | 24269.12 | | | |

| Level | N | Mean | St.Dev |
|---|---|---|---|
| 0-0.05 | 6 | 40.000 | 4.243 |
| 0-0.5 | 6 | 20.167 | 2.787 |
| 0-5 | 6 | 0.000 | 0.000 |
| 12-0.05 | 6 | 39.667 | 4.274 |
| 12-0.5 | 6 | 11.500 | 3.271 |
| 12-5 | 6 | 0.000 | 0.000 |
| 24-0.05 | 6 | 39.500 | 1.871 |
| 24-0.5 | 6 | 19.167 | 2.994 |
| 24-5 | 6 | 0.000 | 0.000 |
| 48-0.05 | 6 | 38.167 | 2.137 |
| 48-0.5 | 6 | 15.000 | 2.608 |
| 48-5 | 6 | 0.000 | 0.000 |
| 72-0.05 | 6 | 39.833 | 2.858 |
| 72-0.5 | 6 | 19.833 | 2.137 |

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